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Novel Food Fermentation Technologies

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K. Shikha Ojha • Brijesh K. Tiwari
Editors

Novel Food Fermentation Technologies

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Editors
K. Shikha Ojha
Food Biosciences
Teagasc Food Research Centre
Dublin, Ireland

School of Food & Nutritional Sciences
University College Cork
Cork, Ireland

Brijesh K. Tiwari
Food Biosciences
Teagasc Food Research Centre
Dublin, Ireland

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Contributors

Saúl Alonso The Centre for Process Innovation (CPI), Wilton Centre, Redcar, UK

C. Ancín-Azpilicueta Applied Chemistry Department, Public University of Navarre, Pamplona, Spain

M. Arias Institute for Global Food Security (IGFS), School of Biological Sciences, Queen's University Belfast, Belfast, Northern Ireland, UK

Francisco J. Barba Faculty of Pharmacy, Nutrition and Food Science Area, Universitat de València, Burjassot, Spain

Sonia Barba-Orellana Centro Sanitario Integrado de Xirivella, Consorci Hospital General Universitari València, Xirivella, Valencia, Spain

Antonio Bevilacqua Department of the Science of Agriculture, Food and Environment, University of Foggia, Foggia, Italy

Carmen Bueno-Ferrer School of Food Science and Environmental Health, Dublin Institute of Technology, Dublin, Ireland

Raffaella Di Cagno Department of Soil Plant and Food Sciences, University of Bari Aldo Moro, Bari, Italy

Maria Rosaria Corbo Department of the Science of Agriculture, Food and Environment, University of Foggia, Foggia, Italy

Mila Emerald Phytoceuticals International and Novotek Global Solutions, London, ON, Canada

Avelina Fernández Instituto de Física Corpuscular (Consejo Superior de Investigaciones Científicas-Universitat de València) Parc Científic UV, Paterna, Valencia, Spain

Pasquale Filannino Department of Soil Plant and Food Sciences, University of Bari Aldo Moro, Bari, Italy

Mariangela Gallo Department of the Science of Agriculture, Food and Environment, University of Foggia, Foggia, Italy

Marco García-Vaquero Food Biosciences, Teagasc Food Research Centre, Dublin, Ireland

University College Dublin School of Agriculture and Food Science, Dublin, Ireland

T. Garde-Cerdán The Vine and Wine Science Institute (La Rioja Regional Government –CSIC – La Rioja University), Carretera de Burgos, Logroño, Spain

Jincy M. George Academy of Scientific and Innovative Research, Central Food Technological Research Institute, Mysore, Karnataka, India

Department of Food Engineering, Central Food Technological Research Institute, Mysore, Karnataka, India

Marco Gobbetti Department of Soil Plant and Food Sciences, University of Bari Aldo Moro, Bari, Italy

Maria Hayes Food Biosciences, Teagasc Food Research Centre, Dublin, Ireland

Dajana V. Hrnjez Faculty of Technology, University of Novi Sad, Novi Sad, Serbia

Mirela D. Iličić Faculty of Technology, University of Novi Sad, Novi Sad, Serbia

Amit K. Jaiswal School of Food Science and Environmental Health, College of Sciences and Health, Dublin Institute of Technology, Dublin 1, Ireland

Katarina G. Kanurić Faculty of Technology, University of Novi Sad, Novi Sad, Serbia

Derek F. Keenan Food Chemistry and Technology, Teagasc Food Research Centre, Dublin, Ireland

Joseph P. Kerry Food Packaging Group, School of Food & Nutritional Sciences, University College Cork, Cork, Ireland

Mohamed Koubaa Sorbonne Universités, Université de Technologie de Compiègne, Laboratoire Transformations Intégrées de la Matière Renouvelable (UTC/ESCOM, EA 4297 TIMR), Centre de Recherche de Royallieu, Compiègne Cedex, France

Vikas Kumar Neuropharmacology Research Laboratory, Department of Pharmaceutics, Indian Institute of Technology, Banaras Hindu University, Varanasi, Uttar Pradesh, India

Begonya Marcos IRTA, Food Technology, Monells, Spain

O. Martín-Belloso CeRTA-UTPV, Food Technology Department, University of Lleida, Lleida, Spain

Spasenija D. Milanović Faculty of Technology, University of Novi Sad, Novi Sad, Serbia

Colm P. O'Donnell School of Biosystems and Food Engineering, University College Dublin, Dublin, Ireland

K. Shikha Ojha Food Biosciences, Teagasc Food Research Centre, Dublin, Ireland

School of Food & Nutritional Sciences, University College Cork, Cork, Ireland

Gaurav Rajauria School of Agriculture and Food Science, University College Dublin, Lyons Research Farm, Co. Kildare, Ireland

Navin K. Rastogi Academy of Scientific and Innovative Research, Central Food Technological Research Institute, Mysore, Karnataka, India

Department of Food Engineering, Central Food Technological Research Institute, Mysore, Karnataka, India

Elena Roselló-Soto Faculty of Pharmacy, Nutrition and Food Science Area, Universitat de València, Burjassot, València, Spain

Samriti Sharma Institute of Medical Microbiology, Hannover Medical School, Hannover, Germany

Milena Sinigaglia Department of the Science of Agriculture, Food and Environment, University of Foggia, Foggia, Italy

Barbara Speranza Department of the Science of Agriculture, Food and Environment, University of Foggia, Foggia, Italy

Brijesh K. Tiwari Food Biosciences, Teagasc Food Research Centre, Dublin, Ireland

Vladimir R. Vukić Faculty of Technology, University of Novi Sad, Novi Sad, Serbia

Chapter 1
Novel Food Fermentation Technologies

K. Shikha Ojha and Brijesh K. Tiwari

1.1 Introduction

The word fermentation is derived from the Latin verb *fevere* which means "to boil" and fermentation was defined by Louis Pasteur as "La vie sans l'air" (life without air) (Bourdichon et al., 2012). Food fermentation has a long history since ancient times which involves chemical transformation of complex organic compounds into simpler compounds by the action of enzymes, organic catalysts produced by microorganisms including yeast, moulds and bacteria (Corma, Iborra, & Velty, 2007). Fermentation is a biotechnological process traditionally used as a means of food preservation and evidences have shown that rice, honey and fruit beverages were produced using fermentation as far back as 7000 BC in China (Marsh et al., 2014). Fermentation processes have been developed for the production of a wide range of products from chemically simple compounds, e.g. ethanol to highly complex macromolecules, e.g. polysaccharides. Recently, fermentation technique has been applied to the production and extraction of bioactive compounds in the food, chemical and pharmaceutical industries. Various processing techniques are applied in conjunction with fermentation process that principally affects a food's physical or biochemical properties along with determining the safety and shelf-life of the fermented product. Consequently, considerable resources and expertise are devoted to the processing technique of healthy and safe products. Alternative or complementary technologies to

K.S. Ojha
Food Biosciences, Teagasc Food Research Centre, Dublin 15, Ireland

School of Food & Nutritional Sciences, University College Cork, Cork, Ireland

B.K. Tiwari (✉)
Food Biosciences, Teagasc Food Research Centre, Dublin 15, Ireland
e-mail: brijesh.tiwari@teagasc.ie

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conventional methods have been employed with varying degrees of commercialization to develop novel fermented food products. The literature suggests that novel technologies can assist food processors to meet both consumer demands for higher quality and safer products and also the industry demand for energy efficient processes (Pereira & Vicente, 2010). The modern fermentation industry is highly competitive and innovative and has been at the forefront in assessing the potential of new technologies to improve fermentation processes and yield better quality products. The food fermentation industry requires novel techniques to improve the productivity and quality of fermented products along with new products from range of food sources.

1.2 History of Fermentation

The relation between fermented food and health dates back from Neolithic Chinese to ancient Roman era and the earliest evidence suggests that fermentation was an integral part of the old civilization. Cheese and breadmaking was purportedly practiced as early as 7000 BC followed by winemaking in 6000 BC. However, it is anticipated that it was Chinese and Georgian who prepared first fermented alcoholic beverages from fruit, rice and honey dates from 7000 to 6000 BC (McGovern et al., 2004). Evidence also suggests that people were fermenting beverages in Babylon, pre-Columbian Mexico and Sudan circa 3000 BC, 2000 and 1500 BC, respectively (Ray & Roy, 2014; Sahrhage, 2008). The production of fermented dairy-based products is mentioned in ancient Sanskrit and Christian scripts, while Romans were the first who revealed the recipe of fermented milk preparation at around 1900 BC. Preparation of fermented meat by Babylonians has been reported to arise during 1000–2000 BC while sausage making was introduced by Julius Caesar into Rome in 48 BC. Preparation of fermented vegetables was first surfaced in China during 300 BC. Undoubtedly, Asian civilizations in particular East Asians have developed a series of fermented food products such as *Lao pa daek* (fish sauce) by Chinese, *Mám* (seafood) by Vietnamese, *Natto* (soybeans) by Japanese and *Banchan* (vegetables) by Koreans for their everyday cuisine. The other fermented foods like pickles, vinegar, sauerkraut, yogurt, cheeses and a number of fermented milk and traditional alcoholic beverages products that were developed by Asians are still popular globally. Furthermore, fermented food such as beer and wine were also used for medicinal purposes and played an extensive role in Asian diets. It was in 1637, when the Gekkeikan Sake Co. begins producing sake (a fermented rice-based alcoholic beverage) in Kyoto, Japan. Despite the long history of fermented food preparation and consumption, the people were unaware about the role of microorganisms, microbial enzymes and their interaction during the process of fermentation. The first breakthrough in this area came when German scientist Korschelt unveiled the role of fungus *Aspergillus oryzae* in the preparation of koji in 1878. The discovery of the role of another fungus *Rhizopus oligosporus*

in fermentation fuelled the research in this area which further triggered the research work on fermentation. Later on, several advancements ranging from the development of various starter cultures for fermentation during early 1900s and genomic sequencing of *Saccharomyces cerevisiae* in 1996 and *lactococcus lactis* in 2001 has revolutionized the fermentation industry.

1.3 Relevance of Fermented Food Products

Fermented food products are currently experienced by every cultural society in the world according to the availability of the food substrate and their food consumption patterns. In many cases, such products play an important role in ethnic identity and culinary enjoyment (Hui et al., 2004). For instance, Europe produces the largest quantity of fermented dairy products while Africa is the largest producer of fermented starch crops and legumes-based food products. Similarly, the fermented fish products are very common in south and south-east Asia whereas North America is presumably the biggest producer of fermented beverages and meat products (Khem, 2009). Over the centuries, fermentation techniques and procedure have evolved, refined and extended which helped some fermented products such as bread, cheese and yoghurt to be produced all over the world. Fermentation is commonly used in the food and functional food industry, and there are approximately 5000 varieties of fermented foods and beverages consumed worldwide (Tamang & Kailasapathy, 2010). Fermented food products include those derived from meat (sausages, salami), dairy (yogurt, cheese and kefir), soy (natto, miso), fruits (wine), cereals (Bread), vegetables (sauerkraut) and fish (surimi). The secondary metabolites produced during fermentation processes range from antibiotics to peptides and are also referred to as bioactive compounds due to their biological activities which are numerous and range from the prevention of chronic diseases such as diabetes and cardiovascular disease to cancer prevention (Limón et al., 2015). Bioactive compounds obtained as a result of fermentation process not only improve the nutritional value of food but also allow shelf-life extension while improving safety profile. Though fermentation has always been an important part of human lives, it was not clearly understood of what actually happens during fermentation until the work of Pasteur in the latter part of the nineteenth century. Over the centuries, fermentation techniques have been refined and diversified for wine making, brewing, baking, preservation and dairy and non-dairy-based fermented products. In addition, apart from artisan taste and historically rich fermented foods, consumers are preferring foods that have beneficial components towards health and wellness. As an important aspect of this trend, probiotic fermented food are getting more attention because of their image as a gut health booster. The increased demand of traditional and/or novel value added fermented products has brought new challenges to the market to develop novel products.

1.4 Overview of the Book

This book aims to provide a comprehensive overview of innovations in food fermentation technologies and application of novel technologies for fermented food products. The unique feature of this book include (1) novel technologies for microbial culture production and preservation; (2) comprehensive overview of novel thermal and non-thermal technologies applicable to fermented food products and (3) novel fermentation techniques for the production of bioactives from various food matrices.

The book contains 15 chapters which include the application of novel technologies for preservation of microbial cultures (Chap. 2) which highlights the relevance of microbial culture and preservation strategies to improve cell viability during storage and use of novel cryopreservation approaches for the development of innovative formulations for microbial preservation. Chapter 3 outlines novel immobilization and encapsulation technologies for safeguarding cell viability and biocompatible within specific food systems. Various novel technologies such as high pressure processing (Chap. 4), pulsed electric field (Chap. 5), power ultrasound (Chap. 6), gamma irradiation (Chap. 7) and novel thermal technologies (Chap. 8) in order to improve safety profile and quality of range of fermented food products are discussed. Second section of this book deals with novel fermented food products from dairy (Chap. 9), meat (Chap. 10), marine (Chap. 11), grains (Chap. 12), fruits and vegetables (Chap. 13) and bioactives from various fermented food products (Chap. 14). Chapter 9–14 provides an overview of range of fermented food products which can be obtained explores their usage through history along with current research trends and future challenges associated with their production. Relevance of fermented products from various food sources and various strategies to improving the image and content of fermented products are also discussed. Maintaining gastronomic value while reduction of ingredients considered unhealthy (such as sodium salt) by smarter use of starter cultures, application of novel processing and development of functional food products are discussed in detail. Role of fermented food products with a capacity to become vehicles for health-promoting compounds, such as probiotics, bioactives and micronutrient producing organisms are highlighted in Chap. 14. Penultimate chapter (Chap. 15) of the book outlines various food packaging strategies and trends applied to the packaging of fermented food products.

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Chapter 2
Novel Preservation Techniques for Microbial Cultures

Saúl Alonso

2.1 Introduction

Over last two decades, the maintenance of structural properties and bioconversion abilities of microbial cell factories during long-term storage has become increasingly important in the industrial manufacturing of functional foods, pharmaceuticals, biofuels, and biochemicals. Nowadays, robust upstream cell propagation schemes are essentially needed to achieve cost-competitive and efficient bio-based processes. As microbial preservation constitutes the first step in any upstream bio-production approach, both cellular propagation and subsequent scale-up processes are influenced by the degree of cellular stability achieved during the post-cultivation and storage stages. In fact, the scenario is featured by an ever-growing development of novel strategies with an aim to ensure both higher storage stability and functionality of microorganisms. Among those upstream operations, cell preservation undoubtedly plays a key role in ensuring a complete and efficient microbial cell propagation, while maintaining metabolite titers, yields, and productivities during any scale-up process.

Within the current bio-economy context, exploiting full microbial capacities while implementing robust upstream processes is of prime importance for achieving cost-effective scalable bioprocesses. In fact, productive degeneration may arise due to non-optimized and unsuitable cell preservation approaches. Loss of cellular functionality through the seed propagation trains has thus prompted the development of novel preservation techniques towards ensuring an optimum cellular stability during long-term microbial storage. Such instability and lack of cellular robustness can undoubtedly be translated into reduced fermentation performances accompanied with unpredictable metabolic responses. As a result, the novel preservation technologies developed during the last decade are playing a key role in preventing microbial productive degeneration

S. Alonso (✉)
The Centre for Process Innovation (CPI), Wilton Centre, Redcar TS10 4RF, UK
e-mail: satuero@gmail.com

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throughout the fermentation process. Furthermore, cell preservation is one of the main challenges ahead of the development and application of efficient probiotics systems for functional foods (Jankovic, Sybesma, Phothirath, Ananta, & Mercenier, 2010). In particular, industrial starter cultures including lactic acid bacteria are sensitive to stressful conditions and the long-term stability of these microorganisms is strongly compromised during their production, storage, and end use. Industrial operations (e.g., freeze drying, spray drying) can negatively influence on the microorganisms' viability and their technological properties. Though many attempts have been made to increase microorganisms' stability during various downstream processing stages, improving the survival rates is still one of the major challenges in industrial starters and probiotics production (Lacroix & Yildirim, 2007). Apart from their long-term storage, cellular stability of probiotics constitutes another challenge to provide beneficial health effects.

Drying and cryopreservation are the most commonly employed techniques for long-term microbial cell preservation. Both preservation strategies entail deleterious impacts on viability, stability, and functionality of microorganisms, hence to achieve balance between stabilization and cell damage is critical in pursuing an efficient cell preservation strategy. Though freeze-drying is widely employed in long-term storage, spray drying has been the chosen technique for microbial dehydration due to its high processing flexibility as well as cost-effectiveness at industrial settings (Schuck, Dolivet, Méjean, Hervé, & Jeantet, 2013). Even though cryopreservation methods are featured by the loss of viability, both cryotolerance and functionality of microbial culture can be improved depending on processing variables employed. In fact, the interaction of factors such as the use of low cooling rates and cryoprotective agents has been the focus of several studies in the last decade with an aim to improve cell viability and long-term stability. Additionally, the use of sophisticated analytical tools has enabled researchers to characterize the physiological cell responses at the single-cell level while understanding the impact of processing strategies on cellular robustness. All these recent advances have contributed to convert microbial cell preservation into an exciting area of research.

This chapter overviews the latest advances in microbial cell preservation along with novel bioprocessing strategies to enhance cellular viability and stability during long-term storage. Technological challenges as well as novel cell preservation methods which can foster the development of functional foods are also discussed.

2.2 Conventional Approaches for Long-Term Microbial Preservation

Efficient drying processes are of prime importance for achieving long-term storage stability since the degree of stabilization of the microbial cultures is directly related to the moisture content. Traditionally, microbial cells have been preserved over long term through cryopreservation or freeze drying (Fig. 2.1). The cryopreservation is the most preferred technique for safeguarding microbial cultures in biological resource centers (Heylen, Hoefman, Vekeman, Peiren, & De Vos, 2012, Peiren et al., 2015). Table 2.1 highlights key advantages and hurdles of some microbial cell preservation techniques.

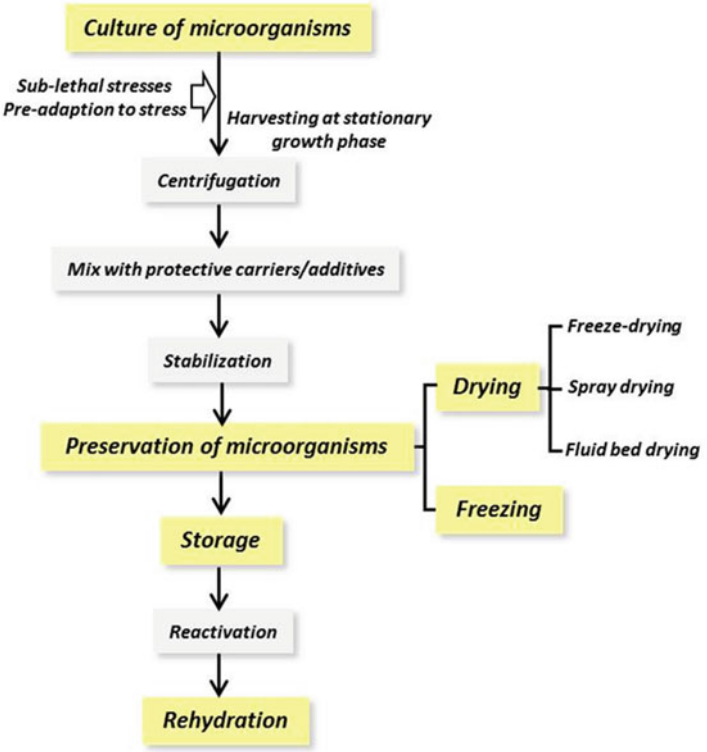

Fig. 2.1 Major processing steps during the cell preservation of microorganisms

Table 2.1 Comparison of main advantages and disadvantages of the techniques used for cell preservation

Process	Advantages	Disadvantages
Cryopreservation	– Low cost protocol	– High energy consumption
	– High success rate	– Storage temperature dependent
	– High cell density	– Cryoprotectants required
Freeze-drying	– Easy handling	– High operating costs
	– Easy transportation	– Complex process
	– Long-term storage	– Cryoprotectants required
Spray drying	– Scalable operation	– Strain dependent
	– Continuous production	– Thermal stresses
	– Cost effective	– Rehydration dependent
Fluid bed drying	– Lower thermal stresses	– Limited strain applicability
	– Rapid heating	– Rehydration dependent
	– Scalable operation	
Microencapsulation	– High survival ratio	– Short-term storage
	– Easy resuscitation	– Storage temperature dependent

2.2.1 Freeze-Drying

Freeze-drying is the most frequently employed technique for drying of microorganisms. The freeze-drying process is based on sublimation which occurs in three phases involving a freezing step followed by two stage drying processes under a high vacuum. Though it is an expensive technique for preserving microbial cells at industrial scale, freeze-drying confers long-term stability without culture transfers, retaining high cell viability after long-term storage periods (Kupletskaya & Netrusov, 2011). As the process involves a freezing step, cellular damages may arise due to the formation of crystals and osmotic stresses. To protect cells against such damages during the freeze-drying a wide range of lyoprotectants, e.g., skim milk, sugars can be added to the drying media before freeze-drying.

When sensitive microorganisms like lactic acid bacteria (LAB) are involved, freeze-drying can even result in the loss of microbial viability and stability in the presence of effective protectants (Carvalho et al., 2004a). To this end, novel methodologies have been sought with the aim of overcoming such limitations. One of those simple approaches combined skim milk and activated charcoal as a carrier material for the long-term preservation of sensitive microorganisms (Malik, 1990). In this way, the good thermal conductive properties of activated charcoal can minimize the freezing degree of the cells during the evacuation process, resulting in a simple methodology to retain a high genetic stability and viability during long-term storage (Malik, 1990).

The choice of the lyoprotectant has a major impact on the storage survival. Thus, standardized freeze-drying protocols for delicate or recalcitrant strains such as *Campylobacter*, *Aeromonas*, and *Vibrio* can result in freeze-dried products of reproducible viability during long-term storage (Peiren et al., 2015). In addition to the importance of the freezing media, characteristics of the cell surface must be taken into account during a freeze-drying process (Otero, Espeche, & Nader-Macías, 2007). Such cell surface features are strain dependent, and are particularly relevant in strong autoaggregative microbial strains. In fact, increased sensibility to a freeze-drying process can be attained due to the large surface area of the cellular aggregates formed when an autoaggregative strain is processed (Otero et al., 2007).

Freezing rate also plays a key role during downstream processes as part of an end-product or to prepare an intermediate product for subsequent freeze-drying process (Volkert, Ananta, Luscher, & Knorr, 2008). The freezing rate affects the location of ice nucleation, size and the growth of crystal, determining the degree of cellular damage of frozen microbial cells (Fonseca, Béal, & Corrieu, 2001). Regardless the low probability of intracellular ice formation, the osmotic-driven migration of water can lead to an increased intracellular solute concentration which can be deleterious for the cells under high freezing rates (Volkert et al., 2008). Generally, higher freezing rates are preferred from economic perspective. Cellular injuries due to the mechanical forces generated by ice crystals can also occur if the freezing rates are high. Feasible alternatives proposed by Volkert et al. (2008)

include using spray freezing to produce a controlled spray of high surface area to volume ratio droplets which rapidly cool down below the freezing point or using pressure shift freezing, which avoids ice formation through a supercooling process in which nucleation occurs instantly.

Innovative microbial preservation technologies involving instant drying steps for the long-term storage without requiring freezing processes have also been described for their application in culture collections. Interestingly, a simple storage system involving of a pre-dried activated charcoal cloth-based matrix within a resealable system was developed by Hays, Millner, Jones, and Rayner-Brandes (2005). The adsorption of the microorganism onto the fibers reduced the stresses exerted to the cells thus improving cell viability upon rehydration (Hays et al., 2005). Though the developed approach seems promising, its wider applicability for drying of various microorganisms has not been investigated.

2.2.2 Spray Drying

Spray drying is the most efficient dehydration technique for the preservation of microbial cultures at industrial scale since it can be carried out in a continuous mode (Peighambardoust, Tafti, & Hesari, 2011). Specifically, the technique involves the evaporation of water through the atomization of a homogeneous solution into a drying chamber. The application of high temperatures is necessary to facilitate water evaporation along the process, and to deliver good storage stability by obtaining final moisture content between 4 and 7 % (Peighambardoust et al., 2011). However, one critical factor in spray drying processes is the high temperatures (85–90 °C) applied during the process, which can lead to heat and osmotic cellular stresses with deleterious impacts on sensitive microorganisms (Ananta, Volkert, & Knorr, 2005). Scalable production has been achieved but stresses generated during the process are still recognized as a major drawback in the applicability of this technology for the large-scale production of microbial dried powders (Fu & Chen, 2011). Though feasibility of spray drying technique is strain dependent, spray drying is recognized as a cost-effective technology in probiotics manufacturing with about ten times lower operational costs compared to freeze-drying (Schuck et al., 2013). Over the last few years, several technical innovations including low heat treatments have emphasized the versatility of spray drying processes in the production of starter cultures and probiotics. In terms of cell viability, the outcome of this technique depends on the addition of protective carrier matrices to offer protection against the high drying temperatures. Carrier matrices like skim milk or polydextrose-based prebiotic substances have been demonstrated to have an effective protection capacity, achieving a cell survival rate of 60 % at an outlet temperature of 80 °C (Ananta et al., 2005). However, the long-term stability of the probiotic cells is affected if polyhydroxylated carbohydrate-based substances are not able to replace water molecules effectively (Ananta et al., 2005).

2.2.3 Fluid Bed Drying

To date, fluid bed drying has been underutilized despite its major potential in cell preservation processes. Specifically, fluid bed drying involves the evaporation of water from wet powder or particles using hot air (Aldabran, Chatzifragkou, Khutoryanskiy, & Charalampopoulos, 2015). Among the advantages of fluidized bed drying, rapid heating and a short drying period, low cost, and easy handling convert fluid bed drying into a promising technology for the manufacturing of stable dried probiotic and starter cultures. However, the lack of extensive research on the cell viability limits its current widespread adoption.

2.2.4 Freezing and Frozen Storage

Cryopreservation remains as the main long-term cell preservation method to date due to its high survival rates. Microorganisms can be cryopreserved at low or ultra-low temperature without genetic or phenotypic alterations while maintaining cell viability (Tedeschi & De Paoli, 2011). Whereas cryopreservation of microbial strains at cryogenic temperatures (<−150 °C) generally results in higher survival rates (Heylen et al., 2012) compared to those stored at −20 °C. Cell viability is severely affected due to the formation of large ice crystals at freezing temperature and can lead to mechanical damage of cell membranes (Tedeschi & De Paoli, 2011). Thus, control of ice crystals is important for improved survival rates.

Among the operating conditions, the temperature, freezing rate, and freezing time play a vital role in maintaining the biological activities during storage. In fact, the lower the temperature and the shorter the duration, the higher acidification activity is preserved in probiotics (Fonseca et al., 2001). Fonseca et al. (2001) reported that cell resistance to freezing and frozen storage can be improved by using a high freezing rate (30 °C/min) and a low storage temperature (−70 °C). Specifically, the freezing rate determines the outcome of the freezing process since mechanical damages may arise due to the presence of ice crystals either inside or outside of the microbial cells. Whereas cellular damages are caused by the extracellular ice accumulation at low freezing rates, high freezing rates have the advantage of forming a glassy rather than a crystallization state (Fonseca et al., 2001). The importance of a homogeneous and earlier freezing process in the cryopreservation of fungal strains has been emphasized by Missous, Thammavongs, Dieuleveux, Guéguen, and Panoff (2007). These authors developed an artificial nucleation and temperature downshift control by adding an industrial ice nucleator protein from biological origin which led to enhanced viability of cells, when subjected to freezing-thawing cycles (Missous et al., 2007).

Despite its major industrial relevance, most fungi cultures are still preserved by repeated subculturing in which a continuous growth is attained by serial transfers

(Homolka, 2014). However, it does not prevent genetic and physiological alterations during long-term maintenance. As a result, cryopreservation at low temperatures has been proposed as an efficient approach to preserve cell functionality (growth, morphology, production of metabolites) in basidiomycetes (Homolka, 2014). Though the genetic stability has been proved, further research is needed since most of the novel protocols entail partial suppression of growth and metabolism of the fungus (Camelini et al., 2012).

In the beverage industry, the long-term maintenance method used for brewer's yeast also plays a key role on maintaining yeast vitality and final beer characteristics (Matoulková & Sigler, 2011). To date, subculturing on agar and cryopreservation have been the preferred approaches for long-term yeast maintenance. Other preservation approaches like freeze-drying are not suitable for long-term maintenance of a brewer's yeast due to the low cell recovery and the viability loss. However, repeated subculturing may lead to time-dependent genetic instability as well as modifications in the flocculation process. Though cryopreservation is the most successful protocol, brewer's yeast cells are sensitive to freeze-thaw stress. To overcome such hurdle, novel cryopreservation protocols have incorporated different levels and types of cryopreservants as well as appropriate equilibration times and cooling rates to prevent membrane damage and the disturbance of cellular organelles (Matoulková & Sigler, 2011). Though the sedimentation ability and viability were not affected in the long-term storage, desired technological properties like the production of beer flavor compounds were enhanced using cryopreserved cells, suggesting the suitability of cryopreservation as a long-term preservation protocol for brewer's yeast cells (Matoulková & Sigler, 2011).

Interestingly, a novel freezing technology called Cell Alive System (CAS) has recently been developed for the immediate preservation of environmental samples (Morono et al., 2015). By applying an alternating magnetic field during the freezing process, a super-cooled liquid phase is created, achieving a uniform freezing process with minimal crystal formation. Such methodology offers stability with minimal loss of viability, suggesting its major potential for preserving fastidious microorganisms (Morono et al., 2015).

2.3 Protective Agents Used in Preservation Processes

The main goal behind any microbial culture preservation technique is to ensure greater cell viability after the downstream processes. Though there is no golden rule behind the formulation of preservation media, it is generally accepted that the inclusion of protectants provides shield against the deleterious effects encountered during cell processing operations and subsequent storage. As a result, cell survival after cryopreservation or dehydration processes can be greatly enhanced by adding extra protective components like cryoprotectants or antioxidants to the media.

2.3.1 Cryoprotective Additives

Microbial preservation has traditionally been carried out by reducing the temperature in order to achieve improved stability. As a rule of thumb, higher microbial viability is preserved at lower storage temperature. If the storage temperature is below the freezing point, cryoprotectants are essential to reduce cell damage from the freezing process. Though loss of viability is inevitable, novel commercial solutions and procedures are already available to minimize the impact of the freezing process and further long-term storage.

Cryoprotectants, also known as lyoprotectants in freeze-drying processes, are additives mixed with the microbial suspensions before freezing to minimize the deleterious influence of ice crystal formation and to lower the freezing point during the freezing processes. Glycerol, dimethylsulfoxide (DMSO), and non-permeable additives like polysaccharides are currently used as cryoprotectants in microbial cultures. These cryoprotective additives are adsorbed to the surface of the microorganisms, coat the cells and therefore provide shield from ice crystals formation during long-term storage. Glycerol conversely acts as a membrane permeant and facilitates the vitrification process by replacing the water in the cells and making hydrogen bonds with water molecules to exert a protective effect (Martin-Dejardin et al., 2013).

Most freeze-drying cell preservation protocols include skim milk as drying medium since it stabilizes the cell membrane constituents by creating a protective coating over the cells (Carvalho et al., 2004a). Protein- and carbohydrate-based matrices have also employed as cryoprotectants. However, the ratio of protection is strain dependent (Hubálek, 2003). The synergistic combination of several cryoprotectants can provide higher protective effects than each component separately (Navarta, Calvo, Calvente, Benuzzi, & Sanz, 2011). With the use of rapidly penetrating agents, both osmotic stress and the formation of extracellular ices are prevented (Hubálek, 2003). Likewise, the presence of antioxidants in the media has been shown to display a protective role by reducing the cryoscopic point of the matrix (Fonseca, Béal, Mihoub, Marin, & Corrieu, 2003). In fact, low ice crystal formation is achieved during freezing and frozen storage when binary and multicomponent solutions including antioxidants (e.g., betaine and sodium glutamate) are included in the protective media (Fonseca et al., 2003). Amino acids can also act as cryoprotectors in *lactobacilli* by increasing the mobility of the fatty acids acyl chains in the membrane core region (Martos, Minahk, Font de Valdez, & Morero, 2007).

The incorporation of unconventional materials to the bacterial suspensions prior to freeze-drying can also help to shield the microbial cells from membrane damage. Recently, in an alternative approach to freeze-drying processes, the use of dry rice cakes has been described as a small-scale and low-tech application for preserving yeasts (Nyanga, Nout, Smid, Boekhout, & Zwietering, 2012). Specifically, the rice starch provided hydroxyl groups for the attachment of the yeast cells, forming a glassing structure and protecting the yeast cells from damage without requiring freeze-drying (Nyanga et al., 2012).

2.3.2 Sugar Preservatives

Sugars have been used for long time as preservatives in freezing and freeze-drying processes due to their ability to replace water during dehydration while maintaining the biological structures in hydrated status (Carvalho et al., 2004a; Hubálek, 2003). In addition to the water replacement ability, sugars are able to form glassy structures which slow down the molecular interactions within the cytoplasm (Hubálek, 2003). Sugars also provide good protection to the microbial cells by replacing the water in the membrane after dehydration and preventing aggregation of proteins by hydrogen bonding with polar groups (Champagne, Gardner, Brochu, & Beaulieu, 1991).

The effect of adding different mixture of ingredients as carriers and thermoprotectants (starch, whey protein concentrate, maltodextrin, etc) in the survival rate is strain dependent. In fact, the intrinsic differences in the glass transition temperatures of such mixtures provide different grade of protection against cell damage (Carvalho et al., 2004a). By using the synergistic combination of skim milk and sugars like sucrose or lactose, the cell viability loss can be reduced after a drying process. In fact, skim milk is responsible to form a protective coating on the cell wall which results in the stabilization of the membrane constituents (King & Su, 1994). Similarly, sugars like trehalose (Li et al., 2011; Nyanga et al., 2012) or lactose (Ananta et al., 2005; Chen, Ferguson, Shu, & Garg, 2011) prevent the formation of ice crystals during drying processes. In addition, the protective effect exerted by polyhydroxylated compounds such as trehalose can be enhanced by adding antioxidants like monosodium glutamate to the carrier medium (Sunny-Roberts & Knorr, 2009). Such synergistic combinations can contribute to maintain not only the membrane integrity and fluidity, but also the enzymatic activity of key metabolic enzymes (Basholli-Salihu, Mueller, Salar-Behzai, Unger, & Viernstein, 2014; Li et al., 2011). However, most of the studies involving the use of cryoprotectants in freeze-drying processes have not demonstrated enough long-term stability (>80 % survival after 1 year) of the freeze-dried bacteria at room or refrigeration temperatures (Corveleyn, Dhaese, Neirynck, & Steidler, 2012). Novel formulations containing alternative cryoprotectants like starch hydrolysate and polyols are being developed to confer long-term stability to the freeze-dried microorganisms (Corveleyn et al., 2012).

In addition to dairy-based carriers such as skim milk, low cost dairy by-products including cheese whey have been proposed as effective growth and protective media. Thus, the formulation of *lactobacilli* media with cheese whey not only provides a potential low cost growing medium but also acts as a cryoprotectant (Burns, Vinderola, Molinaru, & Reinheimer, 2008; Lavari, Páez, Cuatrin, Reinheimer, & Vinderola, 2014). Cheese whey can be also exploited as growth media and encapsulation matrix within a coupled fermentation and spray drying process which avoids the harvesting and resuspension stages found in multistage processes (Jantzen, Göpel, & Beermann, 2013).

Cryotolerance can be induced by choosing the proper medium formulation for growing the microorganisms. Thus, incorporation of sugars like glucose and fructose, as well as polyols like sorbitol in the formulation of growth medium rather

than only in the drying matrix has been found to enhance the protection of *lactobacilli* (Carvalho et al., 2004b; Siaterlis, Deepika, & Charalampopoulos, 2009). However, ultimate protective effect depends on the sugar uptake capacity of the strain since growth medium will not enhance the cell resistance to drying processes unless sugars are transported inside the cell (Carvalho et al., 2004b).

The composition of the drying matrices also plays a key role during freeze-drying of yeast cells. In fact, excipients like maltose and maltodextrins or their mixtures have been found to preserve the viability of *Saccharomyces cerevisiae* cells (Lodato, Segovia de Huergo, & Buera, 1999). The hydrogen bonding capacity of disaccharides plays a critical role in maintain the membrane integrity and protein structures during freeze-drying processes (Lodato et al., 1999).

2.3.3 Galacto-Oligosaccharides and Prebiotic Compounds as Novel Cryoprotectant Agents

Galacto-oligosaccharides (GOS) are polyhydroxylated carbohydrate-based compounds composed by a variable number of galactose units linked to two to eight glucose monomeric units. In addition to their role as prebiotics, GOS have recently gained commercial interest as effective cryoprotectants (Tymczyszyn, Gerbino, Illanes, & Gómez-Zavaglia, 2011). Their protectant capacity is explained on basis to three hypotheses. The first one, known as the vitrification hypothesis, is based on the formation of glassy states which maintains the cells in a vitreous state during storage. The second hypothesis involves the replacement of water by compounds, leading to the interaction between sugars and polar heads of lipids, and decreasing the phase transition temperature of membranes. A third potential mechanism during dehydration-rehydration processes proposes that sugars are excluded from the surface, concentrating water molecules close to the surface and preserving the native structure of the biomolecules (Tymczyszyn et al., 2011). Therefore, the presence of different side chains along GOS structure may present an advantage to interact with biomolecules and to form glassy structures where biomolecules are embedded.

Though the use of polysaccharides with high vitreous transition temperatures does not guarantee appropriate cell preservation. GOS exert great protective capacity due to their high vitreous transition temperatures. In this sense, the synergistic combination of two prebiotic compounds like GOS and lactulose has been found to promote the protection of *lactobacilli* against freezing processes (Santos, Gerbino, Araujo-Andrade, Tymczyszyn, & Gomez-Zavaglia, 2014). The combination leads to the formation of glassy matrices in which molecular interactions are limited due to the high viscosity and low mobility conditions generated (Santos, Gerbino, et al., 2014; Tymczyszyn et al., 2011). In addition to their protective capacity during the dehydration process, GOS mixtures with high content of tri- and tetra-saccharides can also exert a membrane protective role upon rehydration (Santos, Araujo-Andrade, Esparza-Ibarra, Tymczyszym, & Gómez-Zavaglia, 2014). Upon dehydration, GOS stabilize the membrane native structure through the replacement of water

molecules and by forming hydrogen bonds around the polar groups from the phospholipids and proteins (Tymczyszyn et al., 2011). Nevertheless, such protective effect is heavily influenced by the temperature and water content conditions achieved during storage period. In fact, higher survival rates can be attained at lower water content and lower storage temperatures (Tymczyszyn et al., 2012).

In addition to GOS, prebiotics (e.g., inulin and fructo-oligosaccharides (FOS)) have recently been proposed as protective agents of lactic acid bacteria during freeze-drying (Schwab, Vogel, & Ganzle, 2007). In fact, the resulting increased stability and enhanced membrane integrity can be attributed to direct interactions between FOS and the cell membrane, leading to increased membrane fluidity and stability (Schwab et al., 2007). The self-protected symbiotic products generated by supplementing probiotics with compounds like GOS and FOS bear the potential of opening up new commercial applications since such compounds exert both prebiotic and protecting effects.

2.4 Novel Emerging Preservation Technologies

Over the last decade, novel strategies have emerged as complementary approaches to the conventional preservation methodologies. Approaches like microencapsulation or the application of sublethal stresses have emerged as simple and effective strategies to improve cellular properties while preserving the cellular functionality during subsequent processing and storage.

2.4.1 Cellular Immobilization: A Novel Approach for Microbial Preservation

Cellular immobilization has been proposed as an efficient alternative to increase the stability of the microorganisms during the cultivation stage. As opposed to freezing and freeze-drying which may entail irreversible protein denaturation and membrane damages with deleterious effects on cell viability (Carvalho et al., 2004a), microencapsulation has become a feasible technique for shielding the cells while increasing their stability during storage. In general, the effectiveness of encapsulation as cell preservation approach depends on the method as well as the type and concentration of the entrapment material employed. Parameters like size, porosity, and texture also affect the grade of protection exerted to the microorganism (Aldabran et al., 2015). In addition, the nature of the coating material can also promote cell protection as well as increase the effectiveness of the encapsulation process.

Over the last years, microencapsulation has been particularly prolific in the probiotics field since the application of this technique may provide a controlled release of probiotic cells in the human gut under favorable conditions. Though there are several available techniques, extrusion, emulsification, and spray dry-

ing are the three major entrapping techniques for probiotics encapsulation into a gel matrix using an ionotropic gel forming mechanism (Martín, Lara-Villoslada, Ruiz, & Morales, 2015). Recent studies have also highlighted the high storage stability achieved in probiotic cultures through the combination of microencapsulation and dehydration processes (Aldabran et al., 2015). Thus, fluidized bed dried capsuled displayed higher cell survival rates due to the structural collapse and shrinkage observed upon the storage period. Though the protective mechanisms behind the process are not clearly understood, Aldabran et al. (2015) observed an increased agglomeration of the fluidized bed dried powders compared to freeze-dried powders.

Microencapsulation in calcium alginate has been also proposed as an alternative methodology for entrapping probiotic strains, protecting them against freezing temperatures (Sousa et al., 2012). When stored at −80 °C, encapsulation provided a protective effect upon viability in probiotic strains in absence of cryoprotectants (Sousa et al., 2012). In contrast, encapsulation in alginate was not able to exert protection to the encapsulated probiotic cells at −20 °C since major physical changes including larger particle size, loss of spherical shape, and porous net damages were found after a short-term period (Sousa et al., 2015). Interestingly, such results open up the possibility of incorporating probiotics into food matrix that require storage below freezing temperatures without the use of cryoprotectants (Sousa et al., 2012).

Microencapsulation has also been proposed as a feasible approach to increase the cell viability of probiotic strains like *Enterococcus* during drying processes, storage, and gastrointestinal transit (Kanmani et al., 2011). As long-term preservation of entrapped microorganisms requires the dehydration of beads, freeze-drying is usually employed to dry the immobilized beads. However, as previously pointed out, dehydration through freeze-drying involves an oxidative stress which might induce an osmotic shock to the microbial cells. To increase microbial cell protection during freeze-drying, cryoprotective agents are integrated into the entrapment media. As a result, the protective effect exerted by microencapsulation can be also increased by adding trehalose and sucrose (Kanmani et al., 2011) or lactose and trehalose to the entrapment media (Nag & Das, 2013). Moreover, the incorporation of trehalose as cryoprotectant and sodium ascorbate as an antioxidant into the formulation has been found to improve the survival of *bifidobacteria* after freeze-drying (Martin-Dejardin et al., 2013). Thus, the synergistic combination of both compounds leads to replace the water in the cell during the dehydration process while the antioxidant combats the mechanical contraction and osmotic shock during freeze-drying (Martin-Dejardin et al., 2013).

Cell entrapment in inorganic matrices has emerged as alternative methodology for long-term cell preservation without the need for exposing the microorganisms to harsh temperatures. Thus, entrapping cells in a silicon dioxide-derived matrix has been proposed as an effective cell preservation methodology for microorganisms which tend to suffer genetic instability and to lose its metabolite production capabilities during long-term storage (Desimore et al., 2005). Immobilization into a porous

hydrophilic polymer which provides a mechanical strength and thermal stability can be an alternative for long-term preservation of genetic unstable microorganisms.

In addition to the conventional microencapsulation methodologies, immobilization using dry biopolymers has recently become an effective approach to maintain cell viability and functionality during long-term storage (Sorokulova et al., 2012, Sorokulova, Olsen, & Vodyanoy, 2015). This novel microbial preservation methodology allows the cells to be entrapped in water-soluble polymers like acacia gum or pullulan through a spontaneous polymerization and water replacement process which results in the formation of a protective stable film (Sorokulova et al., 2015). As a result, the biopolymer-based film generated is able to protect the microorganisms under several humidity conditions without requiring cold storage (Krummow et al., 2009). In particular, acacia gum seems to trap bound water, preventing complete dehydration of the cell cytoplasm and maintaining the water balance of live cells while increasing their viability (Krummow et al., 2009; Sorokulova, Krummow, Pathirana, Mandell, & Vodyanoy, 2008). This novel cell preservation technology appears to be particularly well suited for preserving spore-forming microorganisms like *Bacillus* without requiring prior time-consuming spore preparation steps (Krummow et al., 2009). Furthermore, spore immobilization in a matrix including acacia gum and porous carriers has led to a 60-fold increase in spore life time at room temperature, suggesting its major potential as long-term spore preservation technique (Sorokulova et al., 2008).

2.4.2 Electrospinning and Electrospraying

High-voltage electrohydrodynamic processes like electrospinning and electrospraying have recently emerged as novel microencapsulation techniques to preserve the viability of sensitive microorganisms. Electrospinning and electrospraying are atomization processes that use an electrically charged jet of polymer solution to form nanoscale and microscale fibers or particles (Fig. 2.2). In addition to their promising food-based applications, both electrospinning and electrospraying have recently emerged as an efficient entrapping methodology for preserving microorganisms due to their high surface area to volume ratios and high permeability (López-Rubio, Sanchez, Wilkanowicz, Sanz, & Lagaron, 2012). Specifically, the thin polymeric fibrous material generated in electrospinning processes allows the entrapped cells to exchange nutrients and metabolic products while retaining their metabolic activity (Liu, Rafailovich, Malal, Cohn, & Chidambaram, 2009) (Fig. 2.2). Both electrohydrodynamic processes can use a wide range of support matrices for cell entrapment, including protein-based materials like whey protein isolate and whey protein concentrate, and polysaccharides like chitosan, cellulose, or alginate (Bhushani & Anandharamakrishnan, 2014). In addition to the versatile use of a wide range of supports, electrohydrodynamic processes offer the advantage of not requiring temperature control, so deleterious impacts on the microbial physiology due to high processing temperatures can be avoided. Such relevant features

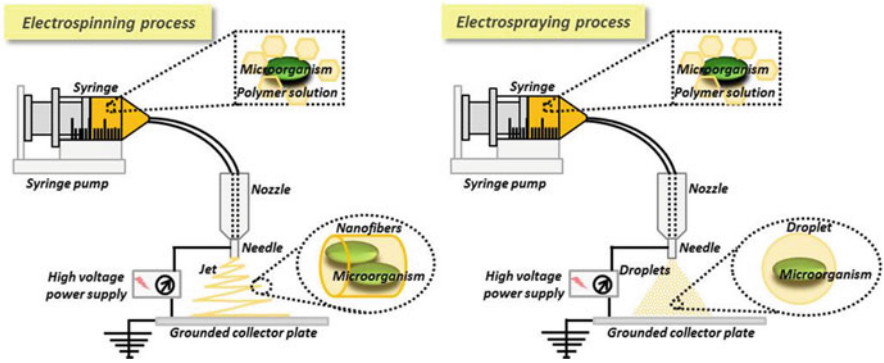

Fig. 2.2 Schematic drawings of typical electrospinning and electrospraying setups for entrapping microorganisms

have converted electrospinning and electrospraying into simple and effective microencapsulation methodologies with potential applications in the development of functional foods (López-Rubio, Sanchez, Sanz, & Lagaron, 2009; López-Rubio et al., 2012).

Recently, electrospinning has been successfully applied for entrapping of bifidobacterial strains by forming electrospun fibers which enabled to maintain high microbial viability despite the drastic osmotic change and electrostatic field generated during the encapsulation process (López-Rubio et al., 2009). Aside from the high cell viability achieved, the incorporation of microbial cells into electrospun nanofibers through electrospinning offers advantages in terms of protein stability and functionally that are hardly achievable with conventional microencapsulation methodologies (Canbolat et al., 2013).

Electrospraying, featured by the atomization of a liquid flow into droplets, has likewise been employed for encapsulating probiotic strains onto protein-based (López-Rubio et al., 2012) or polysaccharide-based matrices (Laelorspoen, Wonsasulak, Yoovidhya, & Devahastin, 2014). Thus, microencapsulation through electrospraying in whey protein concentrate effectively prolonged the survival of bifidobacterial cells even under high relative humidity conditions in comparison to freeze-dried cells (López-Rubio et al., 2012). Microcapsules generated by electrospraying have been able to maintain the viability even under harsh acidic conditions, suggesting an effective probiotics delivery vehicle in the gastrointestinal tract (Laelorspoen et al., 2014).

Though remarkable structural advantages have been obtained by using electrohydrodynamic processes in comparison to conventional dehydration processes, their encapsulation efficiency and long-term stability have not been evaluated so far. Undoubtedly, microencapsulation through electrohydrodynamic processes presents great potential for probiotics in food applications due to their capacity to maintain high cell viability. Nonetheless, further studies on the feasibility for using other probiotic strains and microorganisms as well as optimized operating conditions are required for potential commercial exploitation.

2.4.3 *Use of Stressful Bioprocessing Conditions for Enhancing Microbial Preservation*

Increasing the cellular robustness during the cell propagation stage is of paramount significance in many cell preservation protocols. The application of sublethal stress bioprocessing strategies has lately emerged as an effective approach to enhance cellular robustness during cultivation and prior to downstream processing. Table 2.2 overviews the main bioprocessing strategies used to increase the cell resistance

Table 2.2 Overview of the main bioprocessing strategies employed to improve cell resistance to downstream processes

Microorganism	Bioprocessing strategy	Outputs	References
Lactococcus lactis	Heat/cold shock treatment	Increased cryotolerance	Broadbent and Lin (1999)
		High post-fermentation viability	
	Harvesting time + Suboptimal pH control	Lower acidification activity loss	Velly et al. (2014)
Lactobacillus rhamnosus	Mild heat stresses	Increased post-fermentation viability	Lavari et al. (2015)
	Harvesting time + Suboptimal pH control	Enhanced cryotolerance	Ampatzoglou et al. (2010)
Lactobacillus delbrueckii	Acid adaptation	Enhanced cryotolerance	Streit et al. (2008)
	Suboptimal pH control	Enhanced cryotolerance	Rault et al. (2010)
Lactobacillus buchneri	Osmotic shock stresses	Higher betaine accumulation	Louesdon, Charlot-Rougé, Juillard et al. (2014)
		High survival rate during storage	
Lactobacillus acidophilus	Nutrient starvation	Enhanced cryotolerance	Wang et al. (2011)
Lactobacilli	Mild heat stresses	Better endurance to gastrointestinal digestion conditions	Páez et al. (2012, 2013)
Bifidobacterium bifidum	Sublethal heat shocks	Increased post-fermentation viability	Nguyen et al. (2014)
Bifidobacterium animalis	Suboptimal pH control	Enhanced cryotolerance	Saarela et al. (2005)
Escherichia coli	Nutrient starvation	Enhanced cryotolerance	Gawande and Griffiths (2005)
Saccharomyces cerevisiae	Mild acid shocks	Increased post-fermentation viability	Chu-Ky et al. (2013)
Yarrowia lipolytica	Harvesting time	Increased post-fermentation viability	Pénicaud et al. (2014)

against downstream processes. Though the concept is not new, significant new advances with a particular focus on probiotics manufacturing have come to the fore in the last years (Muller, Ross, Fitzgerald, & Stanton, 2009). Thus, the exposure of microorganisms to sublethal stresses has shown to increase cell viability and resistance against subsequent downstream processes. When cells are exposed to sublethal stresses, repair mechanisms, morphology changes, and excretion of molecules are involved in the cellular response which leads to a higher tolerance against stressful conditions. The frequency and intensity of the stress shocks eventually determine whether positive cross-tolerance mechanisms are induced or not. In this sense, the application of sublethal stresses during the fermentation stage has become an effective strategy to increase cell tolerance.

Among the potential strategies to enhance post-fermentation viability, the application of heat or cold shock treatments has been found to increase the tolerance of starter cultures against freezing and freeze-drying processes (Broadbent & Lin, 1999). Specifically, heat shock (42 °C for 25 min) and cold shocks (10 °C for 2 h) induced changes in the cell membrane lipid composition which resulted in increased cryotolerance and post-fermentation viability (Broadbent & Lin, 1999).

In an interesting approach, stochastic exposure to a sublethal high temperature has been found to improve the cell survival ability of *bifidobacteria* to freeze-drying processes (Nguyen et al., 2014). Cells displayed higher cell resistance to freeze-drying when applied to a sublethal heat shock of 42 °C for a period in a range of 100–300 s during cultivation stage. In fact, such heat shock induced a stress resistance in *bifidobacteria*, featured by the increase in exopolysaccharides synthesis and excretion, which shielded the cells from deleterious impacts during the post-fermentation and subsequent downstream processing stages. As a result, higher cell survival rates upon cell recovery and further freeze-drying steps were obtained in *bifidobacteria* (Nguyen et al., 2014).

Other bioprocessing approaches to increase cell resistance against spray drying processes include the application of mild stresses during cell cultivation. In particular, the heat and oxidative challenges encountered during spray drying of *lactobacilli* were counteracted by the application of a mild heat stress (Lavari et al., 2015). Likewise, the application of mild heat treatment processes (52 °C for 15 min) upon cultivation stage has been found to enhance the survival of *lactobacilli* to spray drying and further post-drying storage (Páez et al. 2013). Such enhanced cell functionality was additionally translated into better endurance to gastrointestinal digestion conditions (Páez et al. 2012, 2013), suggesting that the application of sublethal stresses during the cell cultivation stage may contribute not only to enhance post-drying stability but also to promote the cell resistance of probiotic foods.

Increasing the osmotic stress at the beginning of fermentation has revealed to enhance the cell survival rate of probiotics during storage (Louesdon, Charlot-Rougé, Juillard, Tourdot-Maréchal, & Béal, 2014). Thus, the application of osmotic shocks at the beginning and end of the fermentation can induce the accumulation of osmoprotectants like betaine while maintaining a high acidification activity and survival rate during the storage (Louesdon, Charlot-Rougé, Juillard, et al., 2014). In fact, a 200-fold increase in the viability of freeze-dried *lactobacilli* was found after

applying an osmotic shock with NaCl during cell production stage (Koch, Oberton, Eugster-Meier, Melle, & Lacroix, 2007). Such osmotic shocks not only help to attain a balanced osmotic pressure while preserving the protein functions inside the cells, but also increase membrane fluidity.

In *lactobacilli*, submitting cells to nutrient starvation conditions after cell cultivation has been found to be positive in inducing cryotolerance (Wang, Delettre, Corrieu, & Béal, 2011). The adaptive responses found against the starvation conditions cross-protected *lactobacilli* from cold stresses, enhancing therefore their resistance to freezing and frozen storage. Interestingly, such cross-protection phenomenon entailed an increase of membrane fluidity as well as a stress response involving the upregulation of the proteins involved in carbohydrate and energy metabolisms and pH homeostasis (Wang et al., 2011). Analysis of membrane composition and proteome revealed that the cellular adaptive response in *lactobacilli* starved cells was similar to the freeze-thaw resistance developed by *Escherichia coli* under starvation conditions (Gawande & Griffiths, 2005). Undoubtedly, a rational compromise between starvation conditions and culture production yield must be established to develop a cross-protection phenomenon against drying processes.

Increased viability of *S. cerevisiae* upon freeze-drying processes can also be targeted after applying mild acid stresses to yeast cultures (Chu-Ky, Vaysse, Liengprayoon, Sriroth, & Le, 2013). Chu-Ky et al. (2013) found that the viability of the acid-adapted cells (pH = 3.5) was significantly higher than non-stressed cells. Specifically, the induced cross-protection mechanism involved an increase in the fatty acid saturation degree as well as an intracellular accumulation of reserve carbohydrates in the form of glycogen (Chu-Ky et al., 2013). Similarly, the application of an acid adaptation step before freeze-drying has been found to improve the cryotolerance in probiotic cells (Streit, Delettre, Corrieu, & Béal, 2008). The exposure to an acidic condition (pH = 5.25) for 30 min at the end of the fermentation improved the cryotolerance by inducing a cross-protection phenomenon. In such case, the physiological cell responses were featured by the overexpression of proteins involved in energy metabolism and nucleotide synthesis as well as by the decrease in unsaturated to saturated and cyclic to saturated membrane fatty acid ratios (Streit et al., 2008).

The implementation of specific bioprocessing conditions must be taken into consideration when production and downstream processes are integrated. In fact, cell growth and resistance are strongly affected by the fermentation conditions adopted during the cell production stage (Velly, Fonseca, Passot, Delacroix-Buchet, & Bouix, 2014). Parameters such as pH, harvesting time (e.g., late exponential vs. late stationary growth phase) and fermentation temperature can strongly influence cell resistance upon drying processes. In addition, the nature of the fermentation medium can also include protective compounds. In certain probiotic strains, loss of acidification activity during refrigerated temperature storage can be counteracted by improving the bioprocessing conditions. Thus, the increase in the fermentation temperature along with appropriate pH control strategies and harvesting time at stationary growth phase made possible to reduce the loss of acidification activities during storage at refrigerated temperatures (Velly et al., 2014). Therefore, an enhanced cell resistance can be obtained through a tunable control of the bioprocessing conditions during the production stage.

The harvesting time strongly influences the cell resistance upon processing and storage. In general, cells harvested at the stationary growth phase are more resistant than cells harvested at the exponential growth phase since the former ones develop a general stress resistance. In *lactobacilli*, increasing the harvesting time from early to the late stationary growth phase did not damage the quality of the culture in terms of biomass concentration or acidification activity, but in turn it did have strong impact on the acidification activity (Broadbent & Lin, 1999). The optimization of cell fermentation stage prior cell concentration can therefore confer an advantage against loss of cell viability during dehydration processes.

In yeasts, drying and rehydration processes may not only induce membrane permeabilization, but also result in the loss of metabolic activity (Pénicaud et al. 2014). Such physiological changes have shown to be ameliorated by harvesting the yeast cells at stationary growth phase (Pénicaud et al. 2014). As a result, stationary growth phase cells displayed increased metabolic activities and higher cell viability than cells harvested at the exponential growth phase.

In addition to the harvesting time, cell cryotolerance in probiotics can be induced through a fine-tune pH control during the fermentation stage. As emphasized in several studies, fermentation pH plays a relevant role on the stability of freeze-dried probiotics. In this sense, the implementation of suboptimal pH conditions during the production stage can trigger beneficial stress-induced physiological responses while improving the stability of *lactobacilli* upon cell recovery (Saarela et al., 2005). Thus, cell cryotolerance can be induced by culturing *lactobacilli* at suboptimal pH conditions (pH = 5) in comparison to lower cell functionalities attained under higher pH values (pH = 6) (Rault, Bouix, & Béal, 2010). The impact of combining different fermentation pH control values and harvesting times was also explored by Ampatzoglou, Schurr, Deepika, Baipong, and Charalampopoulos (2010). These authors observed that the acid tolerance and the survival ability of *Lactobacillus rhamnosus* GG during freeze-drying were highly affected by both factors. Whereas late exponential phase cells from pH-controlled fermentations survived significantly better than cells from uncontrolled-pH cultures, late exponential phase cells were more acid resistant regardless of the pH control conditions (Ampatzoglou et al., 2010). Accordingly, fermentation conditions and harvesting time heavily impact on the degree of resistance displayed by the cells upon freeze-drying. In fact, bioprocessing conditions like the accumulation of lactic acid and the reducing conditions reached at the end of *lactobacilli* fermentations can result in major changes in the membrane composition and therefore enhanced cryotolerance (Louesdon, Charlot-Rougé, Tourdot-Maréchal, Bouix, & Béal, 2014).

In *lactobacilli*, the cell resistance to freezing and frozen storage conditions can therefore be strongly affected by the cell physiological state resulting from the bioprocessing conditions implemented during the cell production stage. In contrast, harvesting time has shown no significant impact on the storage stability of freeze-dried *bifidobacteria* (Saarela et al., 2005). Nevertheless, there is supporting evidence that the implementation of sublethal stress strategies as well as optimized bioprocessing conditions can result in enhanced cell cryotolerance.

2.5 Role of Viability in Cell Preservation Techniques

Regardless of the cell preservation technique employed, the maintenance of cell viability across the processing and storage stages is the main goal behind any cell preservation approach. When microorganisms are subjected to dehydration processes, the membrane integrity and fluidity are strongly compromised, leading to a concomitant loss of metabolic activity and viability (Ananta et al., 2005). In freezing processes, the degree of cellular damage including the loss of membrane integrity is influenced by the size of ice crystals as well as location of ice nucleation and crystal growth (Volkert et al., 2008). Membrane damage, and eventually the cell viability, has also been affected by the freezing rate (Cao-Hoang, Dumont, Marechal, Le-Thanh, & Gervais, 2008) or the cold osmotic shock applied during long-term supercooling processes (Moussa, Dumont, Perrier-Cornet, & Gervais, 2008). Therefore, both cell viability and physiological cell responses are intimately linked to the degree of protection achieved during the preservation process.

As both the cell survival ratio and physiological changes play important roles in developing efficient cell preservation protocols, monitoring and characterization such responses at the single-cell level have also been the focus of research in the last decade. Thus, multiparameter flow cytometry has recently appeared as a high-throughput tool to assess metabolic and structural changes at the single-cell level, providing accurate information on injured cell subpopulations that cannot be detected by classical cell counting methods. The application of this technique has therefore enabled not only to quantify the number of *viable but nonculturable* cells but also to determine the changes in the metabolic activity and the membrane integrity of cells subjected to freezing and frozen storage (Chen et al., 2011, Chen, Cao, Fergusson, Shu, & Garg, 2012; Rault, Béal, Ghorbal, Ogier, & Bouix, 2007). Important aspects like the cell resistance of freeze-dried cells against acid stresses (Chen et al., 2011) or the impact of adding different cryoprotectants to the freezing media (Chen et al., 2012) have also been characterized by using multiparameter flow cytometry. Rault et al. (2007) have also employed this high-throughput analytical technique to evaluate the cryotolerance of LAB cultures after freezing and during frozen storage. In addition, the impact of applying lesser deleterious temperatures during fluidized bed drying in *lactobacilli* viability has also been assessed through flow cytometry (Bensch et al., 2014). Such better understanding on the physiological responses of *lactobacilli* at the single-cell level has enabled technical improvements like the use of larger size of fluid nozzles to reduce the mechanical stress during spray drying (Ananta et al., 2005) or the addition of sorbitol to prevent the membrane-associated cell injuries in fluidized bed drying processes (Bensch et al., 2014). Similarly, the information about the membrane damage, depolarization, and metabolic activity of *bifidobacteria* enabled to decipher the real impact of thermal stresses during spray drying at the single-cell level (Salar-Behzadi et al., 2013).

Maintenance of the cell viability upon cell immobilization is paramount to various applications. Flow cytometry-based protocols have been successfully

applied to monitor the cell viability after being microencapsulated (Canbolat et al., 2013) and freeze-dried (Martin-Dejardin et al., 2013). Though cell immobilization can be a useful strategy for cell preservation as it provides a protective microenvironment, the maintenance of high cell viability and high metabolic activity status are not always attained upon cell entrapment (Alonso, Rendueles, & Díaz, 2015).

As previously highlighted, cell viability of *lactobacilli* can be strongly affected by bioprocessing strategies applied during the fermentation stage. In fact, membrane integrity, cell polarization, and metabolic activity of *lactobacilli* are strongly reliant on the pH control or the harvesting time conditions adopted (Alonso, Rendueles, & Díaz, 2014; Rault, Bouix, & Béal, 2008). The implementation of enhanced bioprocessing conditions on basis to the information provided by flow cytometry is therefore important in guaranteeing that microorganisms display maximum cell viability ratios before the downstream processes.

2.6 Challenges Associated to Cell Preservation Techniques in Probiotics

One of the main technological challenges encountered during probiotics manufacturing is ameliorating the cell death while maintaining the cellular functionality during the downstream processes and subsequent storage (Lacroix & Yildirim, 2007). Considering that starters are usually supplied in powdered form, the development of novel formulations containing probiotics needs to meet such requirements (Tripathi & Giri, 2014). In particular, both *lactobacilli* and *bifidobacteria* are very sensitive to process-related stress conditions. In fact, dehydration processes like freeze-drying entail stressful conditions with deleterious impacts on cellular viability. In addition, fine tuning of operating conditions like the freezing rate during freeze-drying processes has limited influence on retaining the full integrity of the cellular membrane. Balancing the optimal freezing rates along with the incorporation of cryopreservants to the freezing media can therefore arise as potential solutions to ameliorate the deleterious effects encountered during freezing processes.

Another important aspect is the impact of the osmotic response in the cell viability during long-term storage. For that reason, the degree of cryotolerance achieved during the cell production stage may pave the way for enhanced functionality upon storage. Strategies like the addition of complementary cryoprotectants to the growth media or the exposure to sublethal stress levels during the fermentation stage may lead to significant improvements in the cryotolerance ability of probiotic cells. Overall, increasing cell viability through the implementation of novel processing strategies as well as the synergistic combination of protective agents and preservation methods constitutes an important step in developing robust probiotics with attractive technological properties.

2.7 Industrial Biotechnology: Attempts to Increase the Viability During Cell Preservation

One of the main drawbacks of large-scale bio-based production lies in the over-time productive degeneration of the microbial cell platforms which can result in uncompetitive bio-production yields. Cellular degeneration with lower metabolite-producing abilities and unstable production profiles may appear if rationale preservation procedures are not implemented. Effective methodologies for culture preservation are important to ensure that the cellular properties and the biosynthetic pathways are not affected during long-term storage. In fact, such long-term genotypic and phenotypic stability will guarantee an optimum post-preservation recovery (Prakash, Nimonkar, & Shouche, 2013). A proper preservation protocol additionally would ensure a short lag phase, maximizing the success of any resuscitation step after long-term storage.

Loss of stability and productive degeneration are among the reasons for productivity failures during the seed train development in industrial bioprocesses. In addition to the instability and loss of productive metabolic capacity of the microbial cells during storage, undesired phenotypes and physiological states may arise due to wrong preservation approaches. In fact, the loss of desired traits constitutes a common feature found in bioprocesses after several repeated transfer stages, with major detrimental effects in the upstream stage during any up-scaling approach. As a result, long-term failures at industrial scale may arise due to a wrong seed train development (Fig. 2.3). Recently, an interesting approach involving the addition of butanol to the storage solution has been developed to prevent productive degenera-

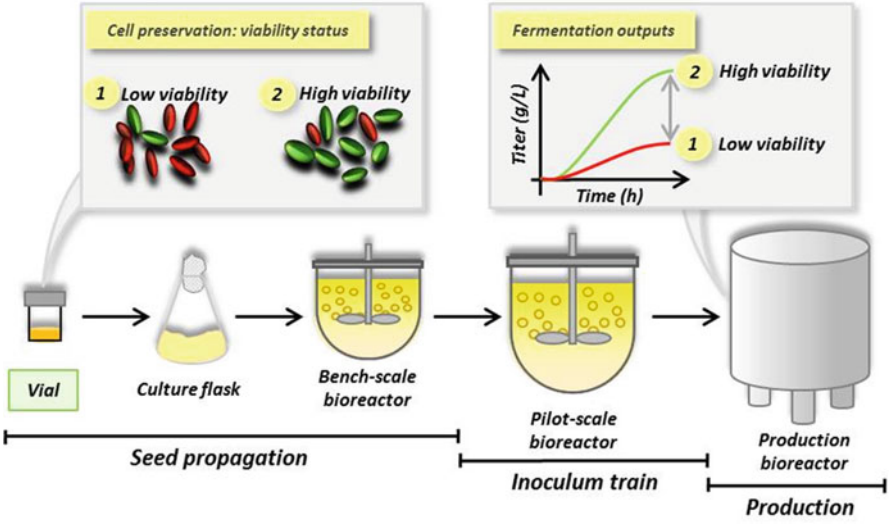

Fig. 2.3 Role of cell viability on microbial production outputs during a typical scale-up process including seed propagation, inoculum train, and final production stage

tion of *clostridia* involved in acetone-butanol-ethanol fermentation processes (Liu, Gu, Liao, & Yu, 2014). Maintaining the cells in a storage solution containing glycerol and butanol at 37 °C not only prevented productive degeneration but also enhanced the cellular robustness of *Clostridium acetobutylicum*. The developed 1-butanol-glycerol storage procedure led to enhanced butanol tolerance, cell viability, and biobutanol productivities (Liu et al., 2014). Alternative methodologies involving the use novel protective formulations have been also explored to prevent productive degeneration in recombinant protein-producing (Desimore et al., 2005) or enzyme-producing microbial cell factories (Pinotti, Silva, Zangirolami, & Giordano, 2007). All these approaches have highlighted the importance of optimizing the cell preservation stage as cornerstone to achieve enhanced productive yields during the scaling-up process.

2.8 Concluding Remarks

The maintenance of cell viability remains as one of the most important challenges in food and industrial biotechnology. Novel cell preservation methods, with higher viability as well as higher stability of the genetic material and metabolism during long-term storage, will definitely pave the way for better yields and production outputs. As a result of better cell preservation processes, robust seed train propagation processes will also enable to enhance the metabolite titers while reducing the failure numbers during the large-scale production at industrial settings.

Likewise, the development of functional foods will be benefited by the advances in the cell preservation field. Undoubtedly, the inclusion of cryoprotective additives is increasingly becoming important in the development of robust dehydration processes for probiotics and starters manufacturing. In particular, the application of novel analytical tools will not only enable to study whether the novel cryopreservation approaches involve changes in terms of physiological and functional properties, but also pave the way for the development of innovative formulations for microbial preservation. Though there are already implemented robust preservation protocols, it is necessary to bridge the gap between the most efficient drying methods and the maintenance of cell viability aiming at taking advantage of the great technological potential offered by probiotics and industrial starters.

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Chapter 3
Novel Microbial Immobilization Techniques

Mariangela Gallo, Barbara Speranza, Maria Rosaria Corbo, Milena Sinigaglia, and Antonio Bevilacqua

3.1 Immobilization and Encapsulation: Introduction

Food industry depends on a constant process of innovation, which follows by the interaction of scientists and engineers, entrepreneurs and consumers. One example of success is the development of the immobilized technology (Kosseva, 2011), that is an exciting field of food industry that has emerged and developed rapidly in the past decade (Mitropoulou, Nedovic, Goyal, & Kourkotos, 2013). The production of microcapsules began in 1950, when Green and Schleicher produced microcapsules dyes by complex coacervation of gelatin and Arabic gum for the manufacture of carbonless copying paper. In 1960, microencapsulation of a cholesteric liquid crystal was proposed through the coacervation of gelatin and acacia for the production of a thermosensitive display material. First proposed for pharmaceutical purposes, then microencapsulation became popular in agriculture, food industry, cosmetics, and energy generation (Dubey, Shami, & Bhasker Rao, 2009).

During recent years, the terms immobilization and encapsulation were used interchangeably. Cell immobilization was defined as "the physical confinement or localization of intact cells to a certain region of space with preservation of same desired catalytic activity" (Kourkoutas, Bekatorou, Banat, & Koutinas, 2004); microencapsulation is an immobilization technique, that has been defined as "the technology of packaging solid, liquid, and gaseous active ingredients in small capsules that release their content at controlled rates over prolonged periods of time" (Champagne & Fustier, 2007). The encapsulated ingredient is called "core material" and is dispersed into matrix that may be called "coating or shell material"

M. Gallo • B. Speranza • M.R. Corbo • M. Sinigaglia • A. Bevilacqua (✉)
Department of the Science of Agriculture, Food and Environment,
University of Foggia, Foggia, Italy
e-mail: antonio.bevilacqua@unifg.it

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(Burgain, Gaiani, Linder, & Scher, 2011). In both cases, the bidirectional diffusion of molecules, such as the passage of oxygen, nutrients, and growth factors, essential for cell metabolism and the outward diffusion of waste products should be permitted (Mitropoulou et al., 2013).

The most important feature of microcapsules is their dimension, i.e., size, thickness, and weight of the membrane; these parameters are usually included in the following ranges:

- Size: 1 μm–2 mm
- Thickness of the membrane: 0.1–200 μm
- Weight of the membrane: 3–30 % of total weight (Dubey et al., 2009)

Many bioactive compounds undergo rapid inactivation or degradation. Thus, they would benefit from an immobilized procedure that slows down and/or prevents degradation. These bioactive compounds include lipids, peptides, vitamins, antioxidants, and living cells such as probiotic bacteria (de Vos, Faas, Spasojevic, & Sikkema, 2010).

Several researchers reported many benefits for microencapsulation, i.e.:

- protection of cells against physicochemical changes, such as pH, temperature, and bile salts
- improved substrate utilization (conversion of liquid active agent into a powder, which might be dust free, free flowing, and might have a more neutral smell)
- higher productivity
- reduced risk for microbial contamination
- controlled release of active compounds over time (Mitropoulou et al., 2013)
- reusability of immobilized cells (Rathore, Desai, Liew, & Heng, 2013)

What are the most appropriate methods and supports for active substance immobilization? There is not a simple answer to this question. The reason is that immobilization technique is a complex process and each application has to be considered one by one, especially when probiotic cells are immobilized to be used in food or beverage industry (Kosseva, 2011). Several biotechnological processes are advantaged by immobilization techniques, therefore numerous methods and support materials have been proposed. There is not an ideal method, but each method of immobilization has specific disadvantages and advantages, consequently a simple and inexpensive immobilization procedure has to be found for each particular application (Kosseva, 2011).

Immobilization techniques mimic nature; in fact, many microorganisms are able to adhere and survive on different kinds of surfaces, and thus cells may grow within natural structures. The immobilization methods can be divided into four categories based on the physical mechanism employed (Fig. 3.1):

1. *Attachment or adsorption* on solid carrier surfaces by physical adsorption due to electrostatic forces or by covalent binding between the cell membrane and the carrier.
2. *Self-aggregation* by flocculation (natural) or with artificially induced cross-linking agents.

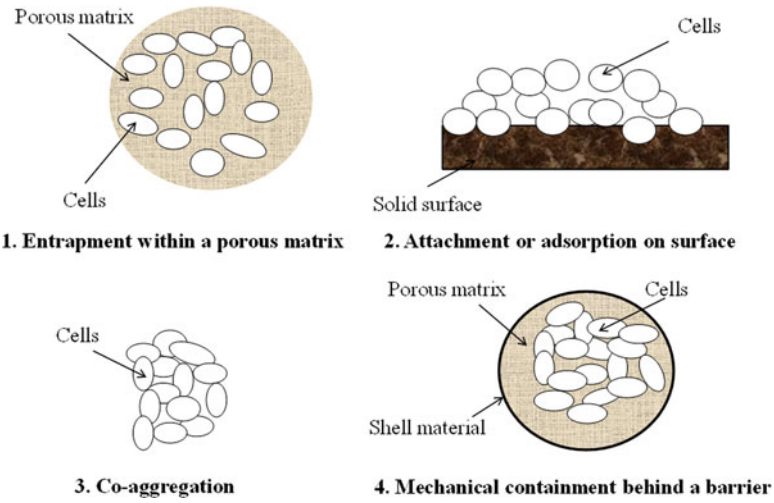

Fig. 3.1 Immobilization methods

3. *Entrapment* within a porous matrix due to cell penetration until their mobility is obstructed by the presence of other cells or to formation of porous material in situ into a cell culture.
4. *Mechanical containment behind a barrier* which could be either a microporous membrane or a microcapsule. Microencapsulation has been used since 1993 as an alternative technology to the entrapment, as it shows the benefit of lacking of binding of active components to the matrix and a higher releasing rate (Kosseva, 2011).

Adsorption utilizes the natural ability of cells to adhere onto solid supports to form microbial biofilm that can exist as a single layer or can be several millimeters thick. For microbial cells that do not adhere naturally, strategies like chemical cross-linking by glutaraldehyde, a silica support, and metal oxide chelation can be used. The advantages of this method include the simplicity and low cost of cell immobilization process. However, it possesses the drawback of an extensive cell leakage from the support, making it difficult to obtain cell-free effluent for downstream processing (Rathore et al., 2013). The ability of some cells, such as yeasts and fungal mycelium, to flocculate, resulting in cell aggregates, has been utilized as a technique of cell immobilization by aggregation. Under some conditions, non-flocculating microbes can also be encouraged to flocculate. Cell immobilization by encapsulation or entrapment involves coating or entrapping microbial cells within a polymeric material to produce small capsules which are permeable to nutrients, gases, and metabolites for maintaining cell viability within the beads (Rathore et al., 2013).

Among all immobilization techniques, encapsulation has found applications in many sectors, including pharmaceutical, agricultural, nutritional, and therapeutics. This technology is currently applied to microbial cell immobilization in order to overcome the drawbacks encountered with other cell immobilization techniques such as limited cell loading, cell leakage, low mechanical stability, contamination, and mass transfer limitations (Rathore et al., 2013).

Table 3.1 Materials used for microencapsulation in the food industry

Origin	Carbohydrate polymer	Protein
Plant	Starch and derivatives	Gluten
	Cellulose and derivatives	
	Gum arabic	
Marine	Carrageenans	
	Alginate	
Microbial/ animal	Xanthan	Casein
	Gellan	Whey protein
	Chitosan	Gelatin

In fact, encapsulation is considered as a mean to prevent viability loss and protect cells against environmental factors (Anal & Singh, 2007; Champagne & Fustier, 2007), and for this reason, the purpose of this method is to create a microenvironment where microorganisms can survive (Kailasapathy, 2006). For microencapsulation of microbial cells, it is essential that the encapsulation process ensures high viability of the encapsulated cells, and good mechanical stability of the capsules.

Many substances have been proposed as suitable carriers for cell immobilization, but not all carriers are suitable for food production; in this case, in fact, carriers have to be characterized by chemical, physical, and biological stability during processing, low cost, high mechanical strength, and high permeability. In addition, these substances must be easily available in nature, with low diffusion problems, nontoxic, biodegradable, and durable (Kosseva, 2011).

For food products, the majority of materials used for microencapsulation are biomolecules. In addition to carbohydrate polymers/polysaccharides, proteins and lipids are also biomolecules suitable for microencapsulation in the food sector (Wandrey, Bartkowiak, & Harding, 2010). Table 3.1 lists the groups of biomolecules, arranged according to their origin, which are found to be most suitable either when used alone or when used in combination with others for microencapsulation in the food industry (Wandrey et al., 2010).

3.2 Innovative Immobilization Techniques: An Overview on the Most Important Applications

Immobilization is successfully used in different biotechnological process fermentation, with the main utilization in the dairy industry (Heidebach, Forst, & Kulozik, 2012). In the following sections we will focus on both classical and innovative approaches for immobilization.

3.2.1 Microencapsulation: New Applications

Several techniques for microencapsulation of microbial cells were studied over the past few years, and some of the most common techniques include extrusion, coacervation, spray drying or freezer drying, and emulsion (de Vos et al., 2010). Table 3.2 shows the benefits and the limits of each approach. Each methodology has its own features and the selection of any particular method is based on the application of the microspheres. Thus, the encapsulation method should be able to produce microspheres with the necessary physical/chemical attributes, causing minimal damage to cell membrane, and the process can be easily scaled up with acceptable costs of process (Rathore et al., 2013).

Since the last 15 years, the protection of probiotics by means of microencapsulation was extensively investigated (Doleyres & Lacroix, 2005). Polymers such as alginate, gellan gum, xanthan, carrageenan, locust bean gum, or their mixtures are commonly used as gelation material that may undergo mild ionotropic and/or thermal gelation also at low concentrations (0.75–4 %). Thus, these materials are commonly applied in many studies dealing with the microencapsulation of probiotic microorganisms (Krasaekoopt, Bhandari, & Deeth, 2004).

Corbo, Bevilacqua, Gallo, Speranza and Sinigaglia (2013) studied alginate beads containing a probiotic strain of *Lactobacillus plantarum*; beads were produced by extrusion and stabilized with $CaCl_2$ solution. They possessed a high encapsulation yield (93 %), and this result appeared of great concern if compared with the data available in the literature; moreover, beads protected cells under simulated gastrointestinal conditions and increased their delivery. In fact, beads released a low amount of cells (ca. 3 log cfu/mL) after 2 h in simulated gastric juice, then, they released a higher amount of cells (7 log cfu/mL) in intestinal juice (3 h), in presence of pancreatin and bile salts. Moreover, Corbo et al. (2013) used the beads as a biocatalyst for the fermentation of soy milk. High viable cell counts were observed throughout 16 h, and pH was reduced below 4.6. Moreover, beads could be successfully used up to ten times, thus assuring one of the basic criteria for a biocatalyst, i.e., the economic feasibility.

Other approaches included alginate–starch (Sultana et al., 2000) and gellan–xanthan beads (Sun & Griffiths, 2000) stabilized with calcium ions. The extent of bead protection relied upon the kind of microorganism, as the viable count of *Lb. acidophilus* loaded into alginate–starch beads was reduced by 5 log of cfu/g at pH 2 for 2 h, while there was a fall in the population of *Bifidobacterium infantis* encapsulated both into alginate–starch and gellan–xanthan beads of ca. 3 log units after exposure under similar conditions (Sun & Griffiths, 2000). Muthukumarasamy, Allan-Wojtas, and Holley (2006) studied the microencapsulation of *Lb. reuteri* in alginate, alginate–starch, k-carrageenan–locust bean gum, and gellan–xanthan beads by both extrusion and emulsion techniques. Their results evidenced that the microencapsulation using alginate or alginate with starch by extrusion or emulsion provided great protection against gastric juice, but the extrusion was the most efficient method.

Table 3.2 Benefits and limits of some microencapsulation methods

Methods	Characteristics	Benefits	Limits
Spray drying	Atomization of a suspension of microbial cells in a polymeric solution into hot drying air, followed by rapid evaporation of water. The microencapsulated product is then separated as a dry powder from the conveying air in a cyclone	Rapid cell release if ingredient dissolves rapidly	High temperature with adverse effects on microorganisms
		Easy to scale-up	More expensive
Emulsion	Dispersion of the cell/polymer suspension in an oil/organic phase. The mixture is homogenized to form a water-in-oil emulsion. Freezing of the dispersed phase is started by cooling or addition of a cross-linking agent to the emulsion	Easy scale-up	Size of the microspheres is affected by the stirring speed and the rate of addition of the cross-linking solution
			Potential toxicity of organic solvents
Extrusion	Polymeric solution is first mixed with microbial cells and then extruded through an orifice as droplets into the solution of a cross-linking agent. Gelation occurs by contact of the polymer solution with the cross-linking agent, cooling or a combination of both	High availability of coating material	Difficult to scale-up
		Simple process	
		High viability of cells	
Freeze spray drying	The process is based upon sublimation: mixture solvent cells is firstly frozen and then dried by sublimation under a high vacuum	Easy to scale-up	Freezing causes damage to cell membrane
Coacervation	Phase separation of one or more incompatible polymers from the coating polymer solution under specific pH, temperature, or composition of the solution was used. The incompatible polymer is added to the coating polymer solution and the dispersion is stirred. Changes in the physical parameters lead to the separation of incompatible polymer and deposition of dense coacervate phase surrounding the core material resulting in formation of microspheres	Good encapsulation capacity and controlled release of core material from the microspheres	High costs
			Control of different critical conditions associated with composition and kinetics of reaction limit

Another interesting approach was proposed by Gallo, Bevilacqua, Speranza, Sinigaglia, and Corbo (2013), who used alginate to produce beads containing *Saccharomyces cerevisiae* var. *boulardii*. In this study, encapsulation yield of alginate beads was ca. 90 %, and cell viability was very high (upon to 90 days); moreover, yeast entrapped in beads survived when exposed to simulated gastrointestinal conditions. Beads were also proposed as a reusable biocatalyst for fermentation of grape juice; results confirmed the suitability of this approach and the possibility of using microspheres to start and guide a fermentation for ten times.

Entrapment of *Lb. acidophilus* within alginate was also proposed for the production of a probiotic tomato juice. This method overcame adverse effects of the tomato juice, and the viable count was maintained upon to 7 log cfu/mL after 10 weeks of storage (King, Huang, & Tsen, 2007).

In recent years, spray drying has been used to encapsulate probiotic cells as an alternative to the encapsulation methods (Heidebach et al., 2012), such as coacervation and fluidized bed coating (Corona-Hernandez et al., 2013). Several carrier materials have been employed (Ananta, Volkert, & Knorr, 2005; Chávez & Ladeboer, 2007; Crittenden, Weerakkody, Sanguansri, & Augustin, 2006; Ying, Phoon, & Sanguansri, 2010). Doherty et al. (2010) and Crittenden et al. (2006) suggested that the presence of a protein in a mixture protects the probiotic against acid stress of gastrointestinal tract.

Recently, water-insoluble hydrogels based on proteins have been successfully applied as a promising alternative to polysaccharide hydrogels (Annan, Borza, & Hansen, 2008; Heidebach, Forst, & Kulozik, 2009). Heidebach, Forst, and Kulozik (2010) investigated the influence of the casein on the viability of probiotic strains (*Lactobacillus* F19 and *Bifidobacterium* Bb12) during freeze-drying and storage. Beads assured cell viability; in fact, the viable count was reduced by 1–2 log cfu/g after 90 days at 4 °C. This kind of effect was not found for starch beads.

The efficacy of whey protein as an encapsulation matrix to prolong the viability of the *Lb. rhamnosus* was proposed by Doherty et al. (2010). After 3 h of incubation in simulated stomach conditions, beads demonstrated high acid stability and peptic resistance.

Saarela, Virkajarvi, and Alakomi (2006) used reconstituted skim milk or sucrose as cryoprotectants during freeze-drying and found that sucrose protected *B. animalis* subsp. *lactis* better than the protein containing formulation in fruit juice. Ying et al. (2013) studied the survival of spray dried microencapsulated *Lb. rhamnosus* GG added into apple juice or citrate buffer (pH 3.5). Capsules containing whey protein or whey protein combined with resistant starch assured a higher cell count.

Table 3.3 reports some examples of encapsulated probiotic bacteria added in different foods during the last 5 years.

Table 3.3 Examples of encapsulated bacteria and their applications in different foods

Product	Probiotic strain	Matrix carrier	Survival of cells	References
Emulsion				
Yogurt-ice cream	*Lactobacillus acidophilus*	Alginate	Count decreased by <1 log cfu/g after 60 days of frozen storage	Ahmadi et al. (2014)
Yogurt	*Lb. rhamnosus*	Alginate	Viability loss was <1 log cfu/g for 4 weeks of storage	Ziar, Gerard, and Riazi (2012)
	Bifidobacterium animalis subsp. *lactis* Bb12	Resistant starch		
Kariesh cheese	*B. adolescentis* ATCC 15704	Rennet-gelled of milk proteins	Viability loss was ~2 log cfu/g after 2 weeks of storage	Abd-Elhamid (2012)
Ice cream	*Lb. casei* Lc01	Alginate	*Lb. casei* decreased by 1.4 log cfu/g	Homayouni, Azizi, and Ehsani (2008)
	B. lactis Bb12	Starch	*B. lactis* decreased by 0.7 log cfu/g after 180 days of storage	
Cheddar cheese	*B. longum* ATCC 15708	Alginate solution	Viability reduction was ~2 log cfu/g after 21 days of storage	Amine et al. (2014)
		Palmitoylated Alginate		
Frozen yogurt	*Lb. casei* 01	Alginate	*Lb. casei* decreased by ~2 log cfu/g	Soodbakhsh, Gheisari, & Aminlari, 2012
	B. lactis Bb12		*B. lactis* decreased of 2 log cfu/g after 150 days of storage	
Spray drying				
Apple juice	*Lb. rhamnosus*	Whey protein and resistant starch	Viability loss was ~2 log cfu/g after 5 weeks storage	Ying et al. (2013)
Yogurt	*Bifidobacterium* Bb12	Skim milk powder and inulin	Viability decreased by 0.25 log cfu/g after 90 days	Pinto, Fritzen-Freire, and Munoz (2012)
Freeze-drying				
Soy bar matrix	*Lb. acidophilu sLA2*	k-Carrageenan	Cell viability was 10^8 log cfu/g after 8 weeks of storage	Chen and Mustapha (2012)

(continued)

Table 3.3 (continued)

Product	Probiotic strain	Matrix carrier	Survival of cells	References
Extrusion				
Pomegranate juice	*Lb. plantarum* NCIMB 8826 *B. longum* NCIMB 8809	Pectin or alginate with double chitosan coating	Depending on the coating: cell count was higher than 7 log cfu/g after 2 weeks	Nualkaekul, Cook, and Khutotyanskiy (2013)
Cranberry juice	*Lb. plantarum* NCIMB 8826 *B. longum* NCIMB 8809	Pectin or alginate with double chitosan coating	Depending on the coating: cell count from 5 to 6 log cfu/g after 2 weeks	Nualkaekul et al. (2013)
Pomegranate juice	*Lb. plantarum*	Alginate with chitosan coating	Viable count from 6 to 8 log cfu/g after 4 weeks	Nualkaekul, Lenton, and Cook (2012)
Ice cream	*B. bifidum*	Alginate	*Lb. plantarum* counts from 9.4 to 8 log cfu/g	El-Sayed, Salama, and El-Sayed (2014, 2015)
	Lb. plantarum	Whey protein	*Lb. casei* counts decreased by 1 log cfu/g	
	Lb. casei	Starch	*B. bifidum* counts from 9.4 to 8 log cfu/g after 90 days of storage	
Cheddar cheese	*B. longum* ATCC 15708	Native alginate Palmitoylated alginate	Viability loss was ~2 log cfu/g after 21 days	Amine et al. (2014)
Orange juice Peach juice	*Lb. paracasei* LAFTI®L26	Alginate or alginate and chitosan or alginate and dextran sulfate	No viability loss after 50 days of storage	Rodrigues et al. (2012)
Berry juice	*Saccharomyces cerevisiae* var. *boulardii*	Alginate Xanthan Inulin	Viability reduction was ~1 log cfu/g after 4 weeks	Fratianni, Cardinale, and Russo (2013)
Yogurt	*Lb. acidophilus* ATCC 4356	Alginate	Viability reduction was ~1 log cfu/g after 28 days	Ortakci and Sert (2012)
Coacervation				
Buffalo milk yogurt	*Lb. acidophilus*	Pectin Casein	Viable count from 9.2 to 8 log cfu/g after 28 days	Shoji, Oliveira, and Balieiro (2013)

3.2.2 Food-Like Production

A new frontier of immobilization is based on the use of natural material. Fruit pieces, gluten pellets, brewer's spent grain, and delignified cellulosic residues were investigated and successfully applied as immobilization supports in wine making, brewing, and whey and milk fermentations (Schoina et al., 2014).

Fruits contain nondigestible carbohydrates, which constitute the base for cell immobilization (Mitropoulou et al., 2013). Fruits were introduced as supports for yeast immobilization, due to ease in the immobilization techniques needed. Apple and quince pieces were considered cheap, abundant supports of food-grade purity of immobilization and they are also compatible with wine aroma and taste. Furthermore, they were found suitable for continuous process (Kourkoutas et al., 2004; Kourkoutas, Kanellaki, & Koutinas, 2006).

A possible way for food-like production was proposed by Gallo et al. (2013), who used *S. cerevisiae* var. *boulardii* as target. Apple pieces of Granny Smith variety, approximately 1×6 cm, were used as support materials. Ten apple pieces were introduced in 250 mL of cell suspension and left to ferment overnight at 30 °C without agitation. Then, the fermented liquid was decanted, and the immobilized biocatalyst was washed twice with distilled water. *S. cerevisiae* var. *boulardii* was adsorbed on the surface of apple pieces, and food-like was used as a biocatalyst for the fermentation of grape juice. Data of this research confirmed what reported by Kourkoutas et al. (2006), who proposed apple pieces as a support of *S. cerevisiae* for wine production and as a reusable biocatalyst for five different batches. *S. cerevisiae* var. *boulardii* showed a similar level of performances, as apple pieces could be used up to seven times to ferment grape juice; moreover, the yield of the system in terms of attached cells was ca. 67 %. Apple and quince pieces proved to be suitable supports for immobilization of *Lb. casei* cells too (Kourkoutas, Xolias, Kallis, & Kanellaki, 2005). The immobilized biocatalysts were used for fermented milk production, and immobilized bacteria could start a new fermentation after storage for 129 days at 4 °C. Immobilized *Lb. casei* cells on fruit pieces have also been successfully used in probiotic cheese production (Kourkoutas et al., 2006).

Recently, fruit and oat pieces were also proposed as vehicles for delivery of *Lb. casei* ATCC 393. The immobilized cells were used for probiotic yogurt production, and cell survival was monitored during refrigerated storage. Microbiological and strain-specific multiplex PCR analysis showed that both free and immobilized *Lb. casei* ATCC 393 were detected at necessary levels for conferring a probiotic effect (at least 6 log cfu/g) for longer periods than required by the dairy industry during storage at 4 °C (Sidira et al., 2013).

Delignified cellulosic materials and gluten pellets were also proposed as natural supports for cell immobilization at both room and low temperatures. These materials showed a significant increase in fermentation rates compared with free cells. Delignified cellulosic materials and gluten pellets are supports of food-grade purity, very cheap, abundant, and easy to prepare industrially, and compared to other natural supports as fruit pieces, they present longer operational stability (Kourkoutas et al., 2004).

Schoina et al. (2014) evaluated *Pistacia terebinthus* (Pafos' Pissa) resin as encapsulation material for *Lb. casei*, and its effect in yogurt was studied. Results showed that immobilization enhanced the viability of *Lb. casei* during storage of the product and the cell concentration was up to 7 log cfu/g, after 60 days. Pafos' Pissa gave a fine aroma and taste, and pathogenic microorganisms were not detected.

During the last years, several encapsulation techniques using cereal fractions have been also tested to improve the viability of the probiotic strains in functional foods. Cereals can be used as fermentable substrates for the growth of probiotic microorganisms; in addition, cereal constituents, such as starch, are expected to have the ability to deliver immobilized probiotic microorganisms to human gut when used as immobilization supports (Mitropoulou et al., 2013).

3.2.3 Yeast Biocapsules: An Example of Natural Immobilization

Regardless of the method of immobilization adopted, cells that have been immobilized in an artificial way are not in a natural state; consequently, not only changes in cell viability may occur, but the metabolic state may be also altered depending on the damage induced by the immobilization procedure. On the other hand, natural immobilization may offer more practical potentiality than artificial methods. Under certain conditions, in fact, different microorganisms can spontaneously bind to a number of supports in a natural way to produce a biofilm where microorganisms are entrapped by extracellular polymers they secrete or to form a flor velum (i.e., the flor yeasts involved in the biological aging of Sherry wines) (Berlanga, Peinado, Millan, Mauricio, & Ortega, 2004). Already in 1986, natural immobilizations of single microorganisms, such as flocculating yeasts, fungal mycelia, and also cell aggregations to form pellets or solid microspheres, have been described (Peinado, Moreno, Villalba, & Mauricio, 2006).

As already mentioned, one of the most critical requirements for successful immobilization of cells is the use of an appropriate material as support, i.e., one that is simple, inexpensive, easy to use, naturally abundant, long-lasting, stable, and fit for nutritional purposes if used by food industry. To ensure successful results, a considerable number of organic and inorganic supports of widely variable origin (alginates, gluten balls, delignified cellulose, porous volcanic rocks) have been proposed as yeast supports for the production of various fermentation products including ethanol, wine, and beer (Baptista et al., 2006; Kandylis, Manousi, Bekatorou, & Koutinas, 2010; Kourkoutas et al., 2004, 2006; Liang et al., 2008; Nguyen, Ton, & Le, 2009; Plessas et al., 2007; Reddy, Reddy, Wee, & Reddy, 2011; Tsakiris, Kandylis, Bekatorou, Kourkoutas, & Koutinas, 2010).

An innovative technique was proposed by Peinado, Moreno, Maestre, and Mauricio (2005) and consisted into the induction of a co-immobilization of a filamentous fungus and a flor yeast without adding chemical cross-linkers or

external supports, but creating special conditions appropriate to induce symbiosis. The novelty of this immobilization technique relied on the use of a living organism (a filamentous fungus) as immobilizing substrate in the absence of inert supports. In particular, the method was designed to enhance the natural, spontaneous co-immobilization of the flor yeast *Saccharomyces cerevisiae* and the filamentous fungus *Penicillium chrysogenum* without the need for an external support or a chemical binder to produce "biocapsules" of the fungus immobilizing the yeast. The spontaneous co-immobilization was forced under appropriate conditions to obtain capsules with walls composed of mycelium and entrapping yeast cells: these walls enclosed an inner space partially occupied by yeast cells that are either free or associated to hyphae to form a cluster.

The specific conditions required for this kind of immobilization were carefully examined by the same research team who developed the technique (Garcıa-Martınez, Peinado, Moreno, García-García, & Mauricio, 2011, 2012; Garcıa-Martınez, Puig-Pujol, Rafael, Moreno, & Mauricio, 2012, Peinado et al., 2005, 2006; Puig-Pujol, Bertran, García-Martínez, & Mauricio, 2013) and could be identified in the following key-points:

1. To stimulate the formation of yeast biocapsules, a carbon source readily available by the filamentous fungus but not by the yeast should be present and added to the medium (for example, gluconic acid). This source makes difficult yeast growth (in fact, only one generation was observed) but not the fungus one. If a carbon source like glucose was instead used, an important growth of free yeast was observed, resulting a medium no suitable for the biocapsules formation.
2. The culture medium should be buffered at neutral pH. Otherwise, the usual excretion of acids by the *Penicillium* leads the yeast cells to die, thus producing lax, fibrous, and rugged spheres composed only of filamentous fungus. Recently, it has been reported that pH plays a very crucial role into the immobilization process, and influences the growth and activity of immobilized microorganisms (Yang et al., 2011).
3. To facilitate the formation of spheres, continuous stirring and aeration of the medium should be assessed to keep the filamentous fungus alive.
4. Another operating parameter for biocapsules formation was the temperature that should be appropriate for optimum growth of the tested microorganisms, i.e., 28 °C.
5. Minimum time for the biocapsules formation was individuated in 7 days; even if the formation of biocapsules can be noticed 3 days after inoculation, only after 7 days the broth was fully transparent and contained no free yeast cells to indicate that all yeast cells were auto-immobilized on the filamentous fungus. If biocapsules were left more time in the medium, they were slightly larger in size due to the growth of the *Penicillium* hyphae and they also showed increased density and consistency. This is an operating parameter to keep in mind, depending on the use of biocapsules (e.g., for the production of sparkling wines it will be necessary that the biocapsules are very consistent to support the high pressures reached in the bottles, 6–7 atm).
6. The optimum concentration of yeast cells for formation of biocapsules was individuated in 4×10^6 cells/mL; at higher concentrations the medium remains turbid for a longer time, and the immobilization process is not suitable.

Thus, if the immobilization process is well performed, the "yeast biocapsules" are smooth and hollow spheres with walls consisting of fungal hyphae and yeast cells that can be reused with no loss of integrity in several fermentation processes. It was observed that the filamentous fungus died during the fermentation process due to a lack of oxygen and remained as a mere inert support facilitating subsequent reuse of the biocapsules (Peinado, Mauricio, & Moreno, 2002).

The forced symbiosis of *S. cerevisiae* and *P. chrysogenum* was the most studied example of spontaneous co-immobilization, even if other filamentous fungi for biocapsules formation (such as *Aspergillus*) have been assayed but discarded because they produced weak, unstable biocapsules unfit for use in fermentation processes (Peinado et al., 2006). Recently, Yang et al. (2011) reported a successful co-immobilization of *Pseudomonas* sp. with *Aspergillus oryzae* to remove malachite green from aqueous solution.

Considering the high practical significance of the yeast *S. cerevisiae*, its potential use in a naturally auto-immobilized form opens new solutions for fermentation. The method not only provides a simple, convenient, inexpensive alternative technique which affords yeast reuse, but since binding and interactions between the two microorganisms are all natural rather than forced on an artificial support (Kourkoutas et al., 2004), the catalytic activity of yeasts was longer preserved. Moreover, the porous nature of the filamentous fungus may reduce diffusion problems with respect to other supports.

In addition to the prolonged activity and stability of the biocatalyst, increased tolerance to high substrate concentration, reduced end-product inhibition, major tolerance against toxic and inhibitory compounds, and stress tolerance are also been recovered in yeast biocapsules (Kourkoutas et al., 2004; Ma & Liu, 2010; Stanley, Bandara, Fraser, Chambers, & Stanley, 2010). These advantages make these biocapsules attractive for the production of ethanol and other alcoholic beverages such as wine or beer; in fact, different applications have been proposed in the last years (García-Martínez, Peinado, Maestre, Moreno, & Mauricio, 2008, Peinado et al., 2006).

For example, yeast biocapsules have been successfully applied as an immobilized cells system in the production of bioethanol from media containing 20 or 40 % molasses, being reutilized up to seven times without observing any decrease in the yield or the rate of fermentation (Peinado et al., 2006). Similarly, biocapsules have been used in a wine fermentation and for the simultaneous saccharification and fermentation process to produce ethanol from starch (Peinado et al., 2006).

In 2012 García-Martínez et al. tested the potential use of wine yeasts immobilized on *P. chrysogenum* for ethanol production. In their study, six different strains of *S. cerevisiae* were used, namely G1, a yeast isolated from the flor film on a wine under biological aging; P29, a strain used in the production of sparkling wines such as cava; X4 and X5, isolates from partially fermented musts from sun-dried grapes and selected for their high osmotolerance; QA23, a selected yeast commercially available; and Uvaferm BC, a *Saccharomyces bayanus* strain selected and marketed as a wine yeast. All the tested strains were from Montilla–Moriles winemaking region (Cordoba, Andalusia, Spain). These six different wine yeast strains were co-immobilized in a natural, spontaneous way

with *P. chrysogenum* under special conditions (Garcıa-Martınez et al., 2011; Peinado et al., 2006) to form six different "yeast biocapsules" that were evaluated in terms of yeast cell viability, ethanol production, and reusability to assess their suitability for ethanol production. The fermentations were realized in a medium containing 18 % (w/v) glucose with repeated fermentations reaching 10 % (v/v) ethanol. X4 and Uvaferm BC biocapsules afforded at least seven uses with no significant decrease in ethanol production; P29 and QA23 biocapsules five times; and G1 and X5 three times. The conclusions highlighted that the strains exhibiting the highest viability and mechanical strength were X4 and Uvaferm BC, i.e., those affording the greater numbers of reuses; these were probably the most suitable for immobilization and use in fermentation processes, even though the other strains may also be effective alternatives, depending on the particular fermentation process.

A recent application of yeast biocapsules was proposed by Puig-Pujol et al. (2013) who studied their efficiency and effectiveness to produce sparkling wines. The performances of biocapsules of *S. cerevisiae*/*P. chrysogenum* were compared with the activity of the same yeast strains in a free cell format and immobilized in calcium alginate beads. Two *S. cerevisiae* strains were used: *S. cerevisiae* P29, a wine yeast from Catalonia vineyards, and a commercial *S. cerevisiae* (Enoferm QA23, Lallemand, Montreal, Canada). Two different base wines were tested. During secondary fermentation, metabolic kinetics of the organic biocatalyst, enological parameters, foam properties, and sensory profile of the produced sparkling wines after 10 months of aging were analyzed. Enological parameters assessed in the final products did not show relevant enological differences, with the exception of calcium ion content, which was slightly higher in sparkling wines made with yeast immobilized in calcium alginate beads. The foaming properties of batches produced with yeast in biocapsules had similar or better values than those fermented with free cells. Results from sensorial analyses reported no significant differences among the final products. In conclusion, even for sparkling wine production, biocapsules stand up as a low cost, natural, and suitable yeast immobilization method.

Although further studies are required to confirm the results obtained in the different previously cited studies, the first results on the use of yeast cells immobilized in biocapsules for production of ethanol and other alcoholic beverages are very promising.

3.2.4 Microcapsules Containing Probiotic Biofilm-Like Colonies

As well recognized, several efforts have been made to enhance the survival of probiotics under the stresses encountered during processing and gastrointestinal transit. Various approaches using microencapsulation of probiotics in different biopolymers originating from seaweed (carrageenan, alginate), plants (starch and

arabic gum), bacteria (gellan, xanthan), and animal (milk, gelatin) proteins (Annan et al., 2008; Guerin, Vuillemard, & Subirade, 2003; Hansen, Allan-Wojtas, Jin, & Paulson, 2002; Rokka & Rantamaki, 2010; Sun & Griffiths, 2000) have been proposed. Small microcapsules have been produced and directly incorporated into foods without affecting their sensory attributes (Chandramouli, Kailasapathy, Peiris, & Jones, 2004; Cui, Goh, Kim, Choi, & Lee, 2000; Hansen et al., 2002; Lee & Heo, 2000; Santillo, Bevilacqua, Corbo, & Albenzio, 2014). Microencapsulation has been found not only to protect the cells from the acidic pH of the stomach, but also to facilitate the gradual cell release in the intestinal gut (Chavarri et al., 2010; Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2011; Guerin et al., 2003; Kanmani, Kumar, & Yuvaraj, 2011).

Although microencapsulation yet provides a physical protection barrier against external stresses, to increase the robustness and viability of the cells, a new approach was recently proposed, i.e., the encapsulation of probiotics in their sessile form (Cheow, Chang, & Hadinoto, 2010; Cheow & Hadinoto, 2013; Cheow, Kiew, & Hadinoto, 2014; Kiew, Cheow, & Hadinoto, 2014). Cells entrapped in biofilm are more robust than their planktonic counterparts, exhibiting a greater resistance to antibacterial agents and various stresses (i.e., heating, freeze-drying, refrigerated storage, and acid) (Cheow et al., 2010), thus this awareness could be advantageously used to increase probiotics viability and resistance. In 2013, Cheow and Hadinoto developed early probiotic microcapsules containing sessile cells of *Lactobacillus rhamnosus*. These capsules were prepared by in situ cultivation of the planktonic cells in the confined space of chitosan-coated alginate capsules. Microcapsules containing biofilm-like colonies of the probiotic strain were prepared by incubation of the planktonic microcapsules in a growth medium supplemented with $CaCl_2$ for a fixed period of 1, 3, or 5 days. The precursor planktonic probiotic capsules were prepared by an external gelation method, using alginate; once constituted, they were hardened in a $CaCl_2$ solution, recovered and re-incubated in a fresh medium where microcapsules containing biofilm-like colonies were allowed to form (Fig. 3.2) (Cheow & Hadinoto, 2013). To enhance their gastrointestinal stability and also to function as a thermal protective layer, the capsules were coated with chitosan (Zorea, 2011). In their earlier studies, Cheow and Hadinoto (2013) proved that the capsules containing biofilm-like colonies exhibited higher freeze-drying resistance (≈40×) and higher thermo-tolerance (>10×), whereas they showed comparable survivals upon acid exposure and during storage, if compared to their planktonic counterparts. On these results, further studies were performed (Cheow et al., 2014; Kiew et al., 2014) and biofilm probiotic microcapsules were produced using alginate mixed with locust bean gum or xanthan gum (*plus* a chitosan coating), in order to pinpoint the better solution performing the optimal gastrointestinal release characteristics and capsule's stress tolerance upon freeze-drying, heat treatment, and acid exposure. Although research is still embryonic, some key points were already pinpointed and could be summarized in the following:

1. The cell densities of the capsules containing biofilm-like colonies were about 7–7.5 log (cfu/mg), regardless the capsule matrix material.

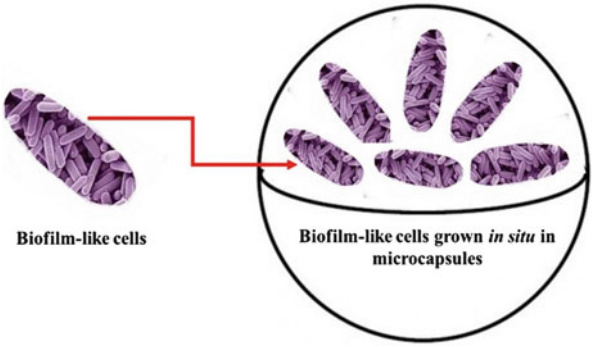

Fig. 3.2 Capsules containing biofilm-like colonies

2. The matrix material resulted fundamental in the acid tolerance. The capsules formed from alginate mixed with locust bean gum, in fact, showed improved freeze-drying viability (≈6×), thermo-tolerance at 100 °C and 45 min (≈100×), and acid tolerance (≈10×) with respect to the capsules formed from alginate alone. The same results were not observed with the capsules made from alginate mixed with xanthan gum, except for the improvement in the acid tolerance (≈5×).
3. The capsule material resulted crucial in cell release profile too: more specifically, the capsules made from alginate mixed with locust bean gum possessed the optimal cell release profile, as a higher number of cells were released in the simulated intestinal juice.
4. The biofilm age and growth medium did not have any significant effects on the capsules' physical characteristics.
5. The biofilm age and growth medium resulted to have a negligible impact on the thermo-tolerance and only a small impact on freeze-drying tolerance, whereas they greatly influenced the cell viability of the capsules during storage and upon exposure to the gastrointestinal juice.

It is worth noting that, despite these promising results, future research is required to optimize this innovative immobilization technique able to improve probiotics viability.

3.3 Conclusion

Immobilization technology has been demonstrated to be an efficient way for maintaining viability and stability of cells, and protects microorganisms during food processing and storage, as well as in simulated gastrointestinal conditions. Conventional methods such as extrusion, emulsion, and spray drying have been

improved through the use of new materials for support of cell immobilization, that must ensure cell viability and biocompatible within specific food systems.

Although research on immobilized cells has been carried out for several years, many difficulties related to the application at industrial scale still exist. In fact, the applicability of these methods for large-scale production still needs to be assessed. Stability of immobilized products is a critical prerequisite for their utilization in various applications, thus it should be carefully considered.

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Chapter 4
High Pressure Processing for Food Fermentation

Jincy M. George and Navin K. Rastogi

4.1 Introduction

The consumer demand for minimally processed foods has always been a challenge for food processing sector. Therefore, novel food processing technologies are implemented to meet the challenges with the aim to ensure safe, fresh-like, nutritive foods without the use of heat or addition of food additives including preservatives. The recent scientific research for the food industry has now focused on non-thermal processing techniques, with high pressure processing (HPP) being amongst the few experiencing great potential in commercial settings (Norton & Sun, 2008; Rastogi & Raghavarao, 2007). High pressure processing is a cold pasteurization technique which consists of subjecting food, sealed in flexible and water-resistant packaging, to a high hydrostatic pressure up to 600 MPa (87,000 psi) for few seconds to few minutes. It is the same effect as subjecting the food to an ocean depth of 60 km deep. The technique was named after Blaise Pascal, a French scientist of the seventeenth century whose work included the effects of high pressure processing on fluids. The history of high pressure processing (HPP) dates back to nineteenth century. In 1899 Bert H. Hite at the West Virginia Agricultural Experimental Station examined the effect of pressure on dairy product like milk, meat, fruits and vegetables. Compared to today's high

J.M. George • N.K. Rastogi (✉)
Academy of Scientific and Innovative Research, Central Food Technological Research Institute, Mysore, Karnataka 570 020, India

Department of Food Engineering, Central Food Technological Research Institute, Mysore, Karnataka 570 020, India
e-mail: nkrastogi@cftri.res.in; nkrastogi@yahoo.com

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pressure processing equipment, the model system (HPP) utilized in the 1890s by Hite was very primitive. Today, with the advancement in techniques like computational stress analysis and new materials, high capacity pressure systems can be manufactured to allow reliable high-pressure treatment of food products at even higher pressures (Hoover, 1993). Experiments into the effects of pressure on microorganisms have been recorded as early as 1884 (Jay, Loessner, & Golden, 2005). In general, high-pressure technology can supplement conventional thermal processing for reducing microbial load, or substitute the use of chemical preservatives (Rastogi, Subramanian, & Raghavarao, 1994). High pressure processing is a natural, environment friendly process with a real alternative to traditional thermal and chemical treatments.

There are two principles applicable to the use of high pressure processing in the food industry. The first is Le Chatelier's Principle, which applies to all physical processes and states that, when a system at equilibrium is disturbed, the system responds in a way that tends to minimize the disturbance (Pauling, 1964). It means with an increase in pressure the volume decreases and vice versa. Second principle is the Isostatic Rule which states that pressure is transmitted instantaneously and uniformly throughout the sample either in direct contact with the pressure medium or hermetically sealed in a flexible packaging material (Olsson, 1995). The pressure applied to food system is transmitted isostatically and instantaneously, irrespective of the shape and size of the food commodity (Smelt, 1998) which offers distinct advantage over conventional thermal processing of large or irregular shaped food products (Kiera et al., 2008).

The fermented food industry is mainly focused on standardizing the properties and extending the shelf life of the product, in order to control the growth of microbes, and to shorten the time-consuming ageing processes required for flavour development. The synergistic effect of high pressure processing and additional hurdles like low pH in case of fermented foods ensures food commodities with better quality attributes and extended shelf life. For example, Bacteriocins work synergistically with HPP inactivating pathogens and increasing their death rate (Galvez, Abriouel, Lucas López, & Omar, 2007). Surviving pressure, cells of the pathogenic bacteria become injured and can be easily inhibited by bacteriocins (Liu et al., 2012). This synergistic action is the basis of the hurdle concept, which implies simultaneous or sequential use of several treatments to achieve product preservation and prolonged shelf life. These treatments include induced changes in aw, pH, temperature and the addition of bacteriocins (Jay et al., 2005). Several reports are available on the use of combination of hurdles with novel techniques like pulse electric field, high pressure processing, irradiation, etc. Hence, an intelligent combination of "hurdles" including non-thermal method of food preservation technique like high pressure processing and competitive microflora may lead to the development of minimally processed foods with added advantage in terms of quality attributes. This approach is described by the phrase "Hurdle technology" (Leistner, 1999; Leistner & Gorris, 1995). The chapter outlines various applications of HPP to extend shelf life of fermented foodproducts.

4.2 Application of HPP for Fermented Food Products

4.2.1 Fermented Meat Products

The meat industry has developed guidelines to control safety of raw meat and other ingredients used in the production of fermented meat products. Some of the measures used to assure the safety of dry fermented sausages involve heating, salting or preservation using vinegar (acetic acid). The use of high temperatures alters the natural characteristics of the product and sensory qualities. Thus, there is a growing need for non-thermal technologies that meet relevant product specifications including improved product safety while maintaining or enhancing sensory characteristics. In HPP, pressure up to 900 MPa is used and its effectiveness on the inactivation of vegetative bacteria in foods has been reported (Aymerich, Picouet, & Monfort, 2008; Considine, Kelly, Fitzgerald, Hill, & Sleator, 2008; Garriga, Grebol, Aymerich, Monfort, & Hugas, 2004; Hugas, Garriga, & Monfort, 2002; Patterson, 2005). Application of HPP in meat processing is widely reported (Considine et al., 2008; Hugas et al., 2002; Patterson, 2005). However, in some cases high-pressure treatment of raw and processed meat can induce lipid oxidation depending on processing parameters of pressure, temperature and time and subsequent storage conditions. Moreover, high pressure can as detrimental as heat treatment in terms of the oxidation level in cold-stored meat products (Simonin, Duranton, & de Lamballerie, 2012). The combination of HPP with biopreservation to enhance bacterial inactivation and reduce the recovery of sublethally injured cells during product storage has been demonstrated in cooked meat products (Aymerich, Jofré, Garriga, & Hugas, 2005; Jofré et al., 2007; Marcos, Jofré, Aymerich, Monfort, & Garriga, 2008).

Shelf-stability of fermented meat products (e.g. sausages) is due to a combination of hurdles, whose interaction inactivates or prevents the growth of undesired microorganisms present in the product (Leistner, 2007). HPP has been reported for various fermented meat products as shown in Table 4.1.

In fermented sausages, high pressure has been proved to be a useful post-process intervention to decrease the levels of several food-borne pathogens (Garriga et al., 2005; Gill & Ramaswamy, 2008; Jofre, Aymerich, & Garriga, 2009; Marcos, Aymerich, Guardia, & Garriga, 2007; Porto-Fett et al., 2010) and has been recognized as a listericidal treatment by FDA and Codex Alimentarius (CAC, 2007; HHS, 2008). Marcos, Aymerich, Garriga, and Arnau (2013) indicated that inactivation of *Enterobacteriaceae* during the whole shelf life of the product could only be prevented with HPP. Pressurization (600 MPa, 5 min) of sliced fermented sausages induced an immediate reduction of *Enterobacteriaceae* counts. Non-pressurized batches showed a 1 log unit reduction of LAB population throughout storage. On the other hand, pressurized batches with or without nisin packed in PVOH films showed reductions of about 2.4 log units at the end of storage. Simon-Sarkadi, Pásztor-Huszár, Dalmadi, and Kiskó (2012) studied the effect of HPP on aerobic plate counts of Hungarian dry fermented sausage during 4 weeks of storage at 8 °C.

Table 4.1 Synergistic effect of HPP and different hurdles on meat products/effect of HPP on various fermented meat products

Product	Conditions	Results	References
Dry-cured ham	HPP: 600 MPa/5 min/15 °C Nisin: 200 AU/cm^2	Increased inactivation of *L. monocytogenes*	Hereu, Dalgaard, Garriga, Aymerich, and Bover-Cid (2012)
Cooked ham	HPP: 400 MPa/10 min/17 °C 1.4 % potassium lactate and 0.1 % sodium diacetate Enterocin: 2400 AU/g	HPP+lactate diacetate: reduced the levels of *L. monocytogenes* during storage at 1 °C by 2.7 log CFU/g; HPP+enterocin: inactivation of *L. monocytogenes* to 4 MPN/g after three months of storage at 1 °C	Marcos et al. (2008)
Sliced cooked ham	HPP: 400 MPa/10 min/17 °C Enterocin: 2560 AU/g	Shelf-life extension to above 90 days	Liu et al. (2012)
Cured pork carpaccio	HPP: 0, 400 and 600 MPa Freezing temperature (−15 °C vs. −35 °C)	HPP in combination with low freezing temperature can be used successfully to deliver high-quality pork carpaccio with extended shelf life	Realini, Guàrdia, Garriga, Pérez-Juan, and Arnau (2011)
Spanish dry sausages (Salchichon)	HPP: 500 MPa/5 min	High-pressure treatment had no noticeable effect on the physico-chemical and sensory properties of the three samples suggesting that it improves the food safety of salchichon with no detrimental effects on organoleptic properties	Rubio, Martinez, García-Cachán, Rovira, and Jaime (2007a)
Reduced fat and sodium low-acid fermented sausage (fuets)	HPP: 600 MPa for 5 min Probiotic strain: *Lactobacillus rhamnosus* CTC1679	The application of HPP at the end of ripening (day 14) produced an immediate reduction of *L. monocytogenes* to levels <1 log CFU/g	Rubio et al. (2014)
Smoked dry-cured ham	HPP: 600 MPa/5 min	Pressurization caused the elimination of *Salmonella* and *L monocytogenes* in ham after 14 days	Stollewerk, Jofré, Comaposada, Arnau, and Garriga (2012a)
NaCl-free processed dry fermented sausages chorizo acid (pH4.8) low-acid (pH 5.2)	HPP: 600 MPa/5 min/13 °C Fast drying	The HPP treatment assured absence of *Salmonella* and *L monocytogenes* in all samples during refrigerated storage for 91 days	Stollewerk, Jofré, Comaposada, Arnau, and Garriga (2012b)
Dry fermented sausage (chorizo)	QDS (Quick-dry-slice), NaCl-free processing, acidification (4.8 and 5.2), smoking and HPP (600 MPa)	Survival of *Listeria monocytogenes* and *Salmonella* was affected by NaCl-free processing, acidification, smoking and pressurization	Stollewerk, Jofré, and Garriga (2013)

Fermented fish sausage	Fast drying HPP: 600 MPa/5 min/13 °C	Pressurization had an important reducing effect on technological microbiota, and eliminated *L monocytogenes, S. enterica*, hydrogen sulphite producing bacteria and *coliforms* immediately after production and during refrigerated storage (4 and 8 °C)	Stollewerk, Jofré, Comaposada, Arnau, and Garriga (2014a)
Dry-cured ham	NaCl-free processing, acidification, smoking, HPP (600 MPa)	The safest hams were those pressurized, especially AS-S hams, where *L. monocytogenes* was eliminated from 25 g of product immediately after HPP treatment and *S. enterica* after 14 days	Stollewerk, Jofré, Comaposada, Arnau, and Garriga (2014b)
Convenience fermented sausage	HPP: 600 MPa/5 min/12 °C PVOH films containing nisin	Combination of HPP with antimicrobial packaging did not produce any extra protection against *L. monocytogenes* compared to antimicrobial packaging alone. The lack of effect of HPP on L. monocytogenes was attributed to a protective effect exerted by the low water activity of the product and its lactate content	Marcos et al. (2013)
Spanish dry fermented sausage "chorizo"	HPP: 350 MPa/15 min/20 °C	Lower levels of tyramine were observed in chorizo and cooked white sausage "butifarra" treated with HP. High concentrations of agmatine were detected in pressurized chorizo	Ruiz-Capillas, Jiménez Colmenero, Carrascosa, and Muñoz (2007)
Restructured dry-cured hams	Potassium lactate and HPP (600 MPa)	The HPP treatment increased significantly the pH, L*, a* and b* values and the breaking stress, and decreased the water-holding capacity and elasticity (apparent Young's modulus) of biceps femoris (BF) muscle	Fulladosa, Serra, Gou, and Arnau (2009)
Genoa salami	Combined effect of fermentation, drying and HPP (600 MPa/ 0, 1, 2, 3, 5, or 7 min, 483 MPa/ 0, 5, 7, 10, or 12 min)	After storage for 28 days at 4 °C, *L. monocytogenes* levels decreased by up to an additional 3.0 log CFU/g, whereas an additional decrease of up to about 1.1 and 1.7 log CFU/g was observed in case of *E. coli* O157:H7 and *Salmonella*, respectively	Porto-Fett et al. (2010)
Low-acid fermented sausage	HPP: 600 MPa/5 min, bioprotective cultures (*Enterococcus faecium* CTC8005, *Enterococcus devriesei* CTC8006 and *Enterococcus casseliflavus* CTC8003)	The application of high hydrostatic pressure (HHP) treatment (600 MPa for 5 min) at the end of ripening (day 21) promoted a decrease of 1 log unit in the counts of *S. aureus*. *E. faecium* CTC8005, which reduced the counts of *L. monocytogenes* ca. 2 log cfu/g immediately after stuffing and in combination with HHP treatment promoted a further reduction of 1 log cfu/g in the pathogen counts	Rubio, Bover-Cid, Martin, Garriga, and Aymerich (2013)

(continued)

Table 4.1 (continued)

Product	Conditions	Results	References
Low-Acid Fermented Sausages (fuet and chorizo)	HPP: 300 MPa/10 min/17 °C	*Salmonella* counts decreased in all studied sausages during ripening at 12 °C and 80 % RH for 27 days. However, the application of HPP as an additional hurdle to the ripening process produced a greater decrease in the *Salmonella* population, showing lower counts (3 MPN/g) in ripened sausages	Marcos, Aymerich, and Garriga (2005)
Norwegian type dry fermented sausages	HPP:600 MPa/10 min 600 MPa/200 s per cycle	The treatment resulted in reduction in *E. coli* by 2.9 $\log_{10}$ CFU/g and 3.3 $\log_{10}$ CFU/g, respectively	Omer et al. (2010)
Fuet	HPP:400 MPa single and combined enterocin AS-48	Drastic 5.5 log cfu/g decrease in *L. monocytogenes*	Ananou et al. (2010)
Fuet and chorizo	HPP:400 MPa /10 min/17 °C starter culture (*Lactobacillus sakei* CTC6626 and *Staphylococcus xylosus* CTC6013)	Sensory analysis showed no differences between lots of chorizo whereas starter fuet was more acid and gummy. High pressure induced an additional reduction of *Enterobacteriaceae* in non-starter sausages. An increase of textural properties was observed after pressurization	Marcos et al. (2007)
Spanish dry fermented sausage "salchichon"	HPP (400 MPa/10 min/12 °C)	Pressure-treated samples showed significantly higher levels of alcohols, aldehydes and alkanes and lower levels of two methyl ketones as compared with control samples	Rivas-Canedo et al. (2009)
Fuets	HPP: 400 MPa Enterocins producing culture: *E. faecium* CTC492	Application of HPP treatment (400 MPa) resulted in reduction of Salmonella less than 1 log CFU/g in all the batches but combination of enterocins and HHP could only reduce the counts of *L. monocytogenes* during storage of the low-acid fermented sausage (fuets) at room temperature and at 7 °C	Jofre, Garriga, and Aymerich (2008)
Spanish fermented sausages (fuet and chorizo)	HPP: 200 MPa/10 min/17 °C Starter culture: *Lactobacillus sakei* (CTC6469 and CTC6626) *Staphylococcus xylosus* (CTC6013 and CTC6169)	HPP prevented *enterobacteria* growth but did not affect Gram-positive bacteria significantly. Subsequently, a strong inhibition of diamine (putrescine and cadaverine) accumulation was observed, but that of tyramine was not affected	Latorre-Moratalla et al. (2007)
Dry-cured ham	**600 MPa /6 min/16** °C	**Absence of *L. monocytogenes* during storage period (120 days)**	**Hereu et al.** (2012)
Sliced beef cured ham	500 MPa/5 min/18 °C	Reduction of *L. monocytogenes* 2 log after 210 days (6 °C)	Rubio, Martínez, García-Cachán, Rovira, and Jaime (2007b)

Salami	483 or 600 MPa/1–12 min	Reduction of *L. L.monocytogenes* numbers by an additional 1.6 to ≥5.0 log CFU g^{-1} when compared to their levels after fermentation and drying; *L. monocytogenes* levels decreased to an additional 3.0 log cfu g^{-1}, during 28 days, under storage at 4 °C	Porto-Fett et al. (2010)
Sliced cooked ham	600 MPa/5 min/10 °C	Reduction of *L. monocytogenes* to levels below 10 cfu g^{-1}	Jofre et al. (2008)
Dry-cured ham	600 MPa/5 min/15 °C	3.5 log inactivation of *L. monocytogenes*	Hereu et al. (2012)
Sliced cooked ham	500 MPa/10 min/25 °C	5 log inactivation of *L. monocytogenes*	Koseki, Mizuno, and Yamamoto (2007)
Turkey breast and ham	600 MPa/3 min/17 °C	3.85–4.35 log reduction in *L. monocytogenes*; during storage remained below detection limit during 154 days	Myers et al. (2013)
Cooked ham	400 MPa/10 min/17 °C	1.9 log reduction *L. monocytogenes* (42 days)	Aymerich et al. (2005)
Dry-cured ham	HPP: 600 MPa/5 min/15 °C	Increased inactivation of *L. monocytogenes*	Hereu et al. (2012)
	Nisin: 200 AU cm^{-2}		
Blood sausages (morcilla de Burgos)	HPP: 600 MPa/10 min	HPP improved the shelf life of morcilla de Burgos to 28 days in comparison with control samples. *Pseudomonas spp.*remained under detection level (b102 CFU/g) after the HPP treatment	Livia, Pásztor-Huszár, Dalmadi, and Kiskó (2012)
Hungarian dry fermented sausages	HPP: 500 MPa/10 min	HPP treatment improved the microbial quality of the sausages and it was effective in the reduction of BA formation during storage (8 °C) for 1 month	Simon-Sarkadi et al. (2012)
Frankfurters	400 MPa, 30 °C, 10 min	Total viable count: 2.16 immediately after processing	Ruiz-Capillas et al. (2007)
Dry-cured ham	Bacteriocin-producing starter cultures and HPP	Shorten drying time	Arnau, Serra, Comaposada, Gou, and Garriga (2007)
Dry-cured fermented ham	400–600 MPa/1.5–20 min	>5 log reduction of *L. innocua* at 600 MPa/20 min, without organoleptic changes	Křepelková and Sovják (2011)

The total viable counts of control samples of semi-dry sausage and extra thick sausage reduced after cold storage, which was followed by regrowth to about 7.5 log CFU/cm^3 within 4 weeks. Treatment with 500 MPa HPP reduced the total viable count by 1–3 log depending on sample type. This difference was more pronounced by the end of storage in case of semi-dry sausage (3 log) and extra thick sausage (5 log). However, significant changes occurred in the organoleptic characteristics of semi-dry sausage. Texture seemed to be the most sensitive to high pressure treatment caused significant changes in firmness of dry and semi-dry sausages, and both samples became softer as a result of pressurization. Smell, firmness and taste of extra thick sausage remained practically unchanged after pressure treatment.

Ananou et al. (2010) demonstrated that in ripened control fuets (a dry-cured sausage), HPP had a low effect on the viability of *Staphylococci* at room or refrigerated storage temperatures. The application of HPP treatment caused an immediate reduction of 1.06 log units in LAB population. However, viable counts recovered to similar values to those of the controls at 18 days storage (at 7 °C or room temperature). Until the end of storage, no significant differences in lactic acid bacteria or *staphylococci* counts were detected between the different types of fuets investigated. However, Rubio, Martín, Aymerich, and Garriga (2014) indicated that the application of high-pressure treatment (600 MPa for 5 min) at the end of ripening (day 14) produced an immediate reduction of *L. monocytogenes* to <1 log CFU/g, which was not detected after 35 days of storage at 4 °C.

Significant changes in volatile profile of high pressure processed fermented meat products have been reported. For example, Rivas-Canedo, Nunez, and Fernández-García (2009) investigated the changes in the volatile profile of Spanish dry fermented sausage "salchichon" when subjected to HPP (400 MPa, 10 min at 12 °C). HPP-treated samples had higher levels of aldehydes (both linear and branched chains), alcohols and some compounds e.g. 1-methoxy-2-propanol, carbon disulfide and benzaldehyde. Similar changes in volatile profile of pork meat have been reported for high-pressure processed samples (Rivas-Canedo, Ojeda, Nuñez, & Fernández-García, 2012).

Changes in biogenic profile of high-pressure processed fermented meat samples have been reported extensively. For example, Latorre-Moratalla et al. (2007) investigated the effects of high hydrostatic pressure (200 MPa) on meat batter just before sausage fermentation and the inoculation of starter culture to improve the safety and quality of traditional Spanish fermented sausages (fuet and chorizo). In batches without starter culture and no HPP treatment biogenic amine accumulation was much lower in fuet compared to chorizo sausages. Tyramine was the only biogenic amine detected in fuet, whereas cadaverine was the major amine in chorizo sausages, which is usually associated with lysine-decarboxylase activity of undesirable Gram-negative bacteria. In spontaneously fermented sausages, the application of HPP resulted in a strong inhibition of diamine accumulation, the levels of putrescine and cadaverine being up to 88 and 98% lower than the non-pressurized batch as shown in Fig. 4.1.

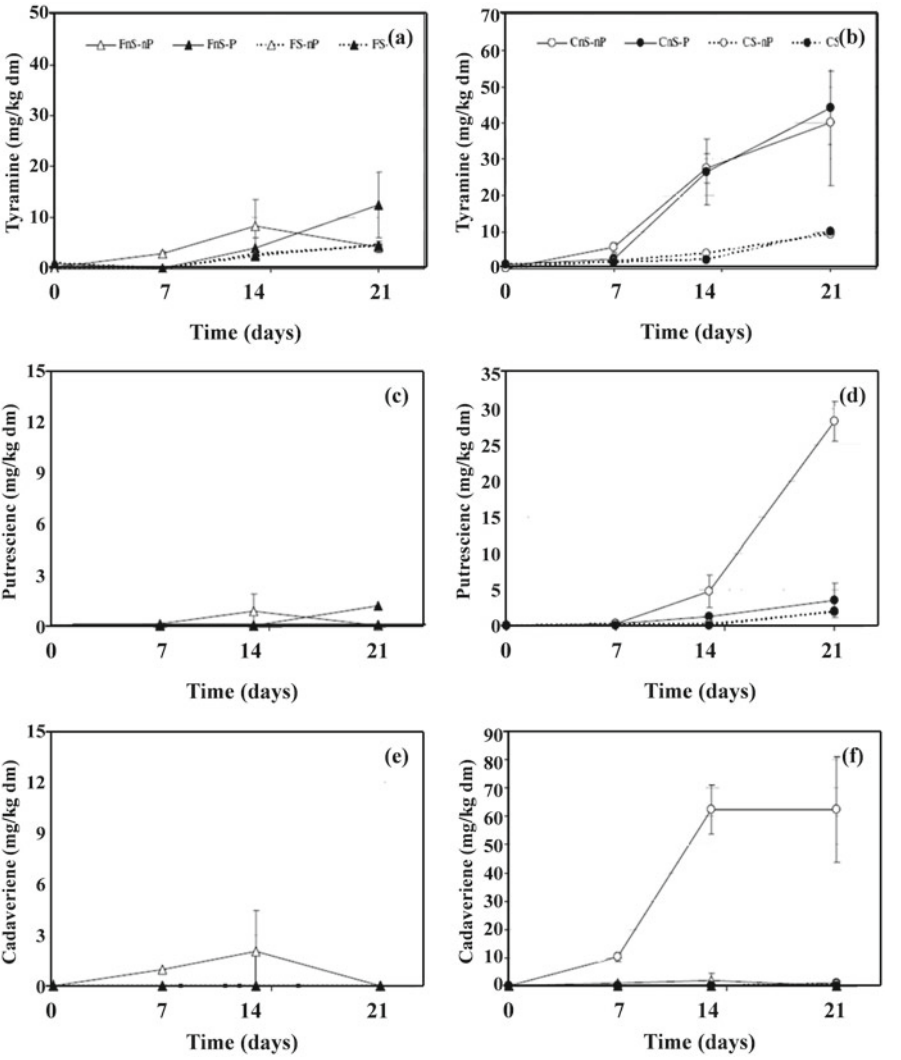

Fig. 4.1 Variation in biogenic amine contents during the manufacture of fuet (**a**, **c**, **e**, *left column*) and chorizo (**b**, **d**, **f**, *right column*) through spontaneously (nS) and starter (S) mediated fermentation, without (nP) and with (P) high hydrostatic pressure treatment (From Latorre-Moratalla, M.L., Bover-Cid, S., Aymerich, T., Marcos, B., Vidal-Carou, M.C., Garriga, M. 2007. *Meat Science*, 75: 460–469. With permission)

4.2.2 Fermented Dairy Products

The dairy industry is focusing on standardizing the properties and extending the shelf life of the milk products. Today, there has been a strong demand in the consumption of probiotic bacteria using food products, including probiotic dairy products due to increasing consumer awareness about the impact of food on health.

Fermented milk is the most popular and most consumed probiotic food carrier throughout the world. Several studies about the development of probiotic fermented milks (Almeida et al., 2008; Kearney et al., 2009; Oliveira, Perego, Converti, & Oliveira, 2009) and probiotic fermented beverages (Castro, Cunha, Barreto, Amboni, & Prudêncio, 2009; Zoellner et al., 2009) have been reported. Pasteurization of milk for the destruction of pathogenic microorganisms and to kill the natural microflora has been traditionally carried out by heat treatment. The potential of HPP treatment as an alternative method to heat pasteurization of milk was proposed almost a century ago (Hite, 1899) and has been investigated for a range of dairy products Apart from pasteurization effects, HPP provides new opportunities for homogenization effects. Table 4.2 summarizes various studies highlighting the application of HPP in

Table 4.2 Synergistic effects of HPP and different hurdles on dairy products

Product	Conditions	Results	References
Raw-milk cheese	300 MPa/10 min/10 °C 500 MPa/5 min/10 °C Bacteriocin:10^6 CFU/mL	*E.coli* O157:H7 counts to levels below 2 log units in 60-day-old cheeses	Rodriguez, Arques, Nunez, Gaya, and Medina (2005)
Milk	250–350 MPa/5 min/20 °C Nisin: 0–500 IU/mL	Treatment at 400 MPa for 5 min with 500 IU/ml nisin, or at 250 MPa for 5 min with 500 IU/mL nisin, gave $a>8$ log reduction of *E. coli* and *P. fluorescens*, respectively	Black, Kelly, and Fitzgerald (2005)
Yoghurt	Supplemented Probiotic bacteriae *Lactobacillus Acidophilus* and *Bifidobacterium* spp., using HP processing	Cultures maintained populations of 10^6–10^7 log CFU/g during a 4 week storage at 4 °C	Jakowska, Wisniewska, and Reps (2005)
Probiotic Fermented milk	HPH: (60 MPA, one homogenized stage alone or combined with 90 °C/30 min heat treatment) *Probiotic bacteria:L. paracasei* and *L. acidophilus*	Survival of cultures 7.0 log CFU/g	
Blackberry yoghurt	HPP: 550 MPa/10 min/4 °C	The treatment with HPP inactivated yeasts and moulds and no growth was observed following prolonged storage at 25 °C	Walker et al. (2005)
Probiotic low-fat yoghurt	HPP:676 MPa/5 min Thermal treatment:85 °C/30 min	The combined HPP and heat treatment of milk before fermentation, and after inoculation of 0.1 % (for both cultures), resulted in a creamy and thick consistency texture which does not require the addition of stabilizers	Penna et al. (2007)

(continued)

Table 4.2 (continued)

Product	Conditions	Results	References
Milk	HPP:345 MPa Bacteriocin (5000 AU/mL sample)	Combination of bacteriocin and HPP treatment resulted in more than 8 log cycle reduction in cell population of *S. aureus* and *L. monocytogenes* was achieved in milk samples stored at 25 °C upto 30 days	Alpas and Bozoglu (2000)
Yoghurt	HPP: 400–600 MPa	Yoghurt processed at a pressure of 550 MPa maintained its beneficial sensorial characteristics longer than the non-pressurized yoghurt, during storage for 4 weeks at refrigerating (4 °C) and room (20 °C) temperatures. The pressure prevented the post-acidification in the yoghurt	Jakowska et al. (2005)
Yoghurt	HPH: 300-750 bar	HPH milk treatment enhanced the viability of *S. thermophiles* and *L. delbrueckii spp. bulgaricus* during refrigerated storage of yoghurt and favoured the growth of the former, compared to that of the latter, thus reducing the risk of post-acidification	Lanciotti, Vannini, Pittia, and Guerzoni (2004)
Goat's milk cheese	HPP: 50 MPa/72 h, 400 MPa/5 min and 400 MPa/5 min followed by 50 MPa/72 h all at 14 °C	By measuring proteolysis indexes, 400 MPa treatments were found to accelerate ripening (14 days in contrast to 28 days conventionally) due to enhanced enzyme activity from inoculated starter culture. pH was higher in 400 MPa treatments compared to the other treatments	Saldo, Sendra, and Guamis (2000)
Goat's milk cheese ripening	HPP: 500 MPa at 20 °C for 15 min	Organic acid levels of cheese made with pressure-treated goat's milk rose gradually for 60 days	Buffa et al. (2001)
Curd formation and firming	HPP: 100–800 MPa/0–60 min	High-pressure treatment up to 250 MPa did not result in any change in curd yield but the moisture content decreased by 5 % in comparison with untreated milk	Huppertz, Fox, and Kelly (2004)

(continued)

Table 4.2 (continued)

Product	Conditions	Results	References
Gorgonzola cheese rinds	400–700 MPa/1–15 min	High-pressure treatment was effective in reducing of *L. monocytogenes* on Gorgonzola cheese rinds without significantly changing its sensory properties	Carminati et al. (2004)
Fermented milk	HPP: 300 and 600 MPa/5 min and pH (4.30, 5.20 and 6.50)	The rate of proteolysis in milk samples at pH values of 5.20 and 6.50 during storage was significantly reduced by treatment at 600 MPa. Treatment at 600 MPa also reduced the viable counts of both Candida yeast species to below the detection limit (1 CFU mL^{1}) at all pH levels for the entire storage period	Daryaei, Coventry, Versteeg, and Sherkat (2010)
Goat milk cheese	HPP: 400 MPa or 600 MPa/7 min)	Treatments at 600 MPa at the three stages of cheese maturation decreased the counts of undesirable microorganisms in mature Ibores cheese (day 60), such as psychrotrophic bacteria, *Enterobacteriaceae* and *Listeria* spp. Mature cheeses (day 60) pressurized at the beginning of ripening showed a higher variation of texture profile analysis. In the sensory analysis, cheeses treated at day 1 showed a significant change of appearance, odour and texture	Delgadoa, González-Crespoa, Cava, and Ramírez (2012)
Starter-free fresh cheese	500 MPa (5 min,16 °C)	The results showed that pressurized cheeses presented a shelf life of about 19–21 days when stored at 4 °C, whereas control cheese became unsuitable for consumption on day 7–8. On the other hand, cheese treated at 500 MPa was firmer and more yellow than the untreated one	Evert-Arriagada et al. (2014)

(continued)

Table 4.2 (continued)

Product	Conditions	Results	References
Fresh cheese	HPP: 300 and 400 MPa for 5 min at 6 °C	Cheeses treated at 300 and 400 MPa, stored at 4 °C, presented a shelf life of 14 and 21 days, respectively, compared to untreated control cheese, which presented a shelf life of 7 days. On the other hand, HP treatments modified the texture (more firm) and colour (more yellow) compared to control cheeses	Evert-Arriagada et al. (2014)
Rennet-coagulated soft cheese	HPP: 291 MPa/29 min	Fat content increased apparently as moisture decreased significantly after HP treatment of above 100 MPa. Increased pressures reduced lipid oxidation but increased yellowness although the latter showed more effect over redness in the HP-treated fresh cheese. Also, increased pressures increased hardness and decreased acidity and adhesiveness in HP-treated fresh cheese	Okpala et al. (2010)
Stirred yoghurt from reconstituted skim milk	HPP treated at 100, 250 or 400 MPa, at 25, 70 or 90 °C, for 10 min prior to starter culture inoculation	HP treatment of skim milk at 25 °C, before or after heat treatment, gave stirred yoghurts of similar viscosities to that made from conventionally heat-treated milk. Lower viscosities were obtained when stirred yoghurts were made with milk HP-treated at elevated temperatures	Udabage et al. (2010)
Reconstituted cow milk	HPP: 200 and 600 MPa, 0 and 25 min, and heat treatment 15 °C and 65 °C, respectively	Particle size distribution of the heat-assisted high-pressure treated milk showed that the number of fat globules below 1×10^{-6} m increased as pressure and pressurization time increased	Sahu and Mallikarjunan (2012)
Hispánico cheese	HPP: 200 and 300 MPa	The use of ewe raw-milk curd pressurized at 200 and 300 MPa, stored frozen and thawed for Hispánico cheese manufacture had increased cheese yield because of the lower dry matter content	Alonso, Picon, Gaya, Fernández-García, and Nuñez (2012)

(continued)

fermented dairy products. Patrignani et al. (2007) evaluated the effects of various factors like milkfat content, non-fat milk solids content, and high-pressure homogenization on rate of fermentation of the probiotic strain *Lactobacillus paracasei* BFE 5264 inoculated in milk. Loss of *Lactobacillus paracasei* strain during refrigerated storage, texture parameters, volatile compounds and sensorial properties of the coagula obtained were investigated. Patrignani et al. (2007) observed significant effect of independent variables on the measured attributes of fermented milks. The coagulation times were significantly affected by pressure and added milk fat, and the rheological parameters such as firmness, viscosity index and consistency of the fermented milk increased with the pressure applied to the milk. Rodriguez-Alcala et al. (2015) indicated that neutral and polar lipids remained stable in the pressure treated sample (250–900 MPa).

Consumer demand for shelf-stable yoghurt is difficult due to problem of storing live microflora at ambient conditions. The use of heat treatments for extension of shelf life of yoghurt has resulted in syneresis and a decrease in viscosity during storage. The application of hydrostatic pressure directly to yoghurts has been proposed as an alternative to the use of additives, which can adversely affect the yoghurt taste, flavour, aroma and mouthfeel (De Ancos, Cano, & Gomez, 2000). Penna, Rao-Gurram, and Barbosa-Cánovas (2007) investigated the effect of HPP on microstructure of low-fat probiotic yoghurt. Microstructure of HPP (C and D) processed yoghurt has more interconnected clusters of densely aggregated protein of reduced particle size, on the other hand heat-treated (A and B) milk yoghurt had fewer interconnected chains of irregularly shaped casein micelles (Fig. 4.2).

Cheese is one of the most versatile dairy products available and consumed in various forms. Application of HPP has shown to improve shelf life and safety profiles of cheese while inducing desirable changes in techno-functional and organoleptic properties. Evert-Arriagada, Hernández-Herrero, Guamis, and Trujillo (2014) demonstrated the commercial application of HPP to increase the starter-free fresh cheese shelf life. High-pressure treatment of cheese did not affect panellists' preference for treated cheese over the non-treated cheese. Moreover, the preference mean score (6.5) for the pressurized cheeses stored during 22 days and for the freshly made cheese was almost the same. Bermúdez-Aguirre and Barbosa-Cánovas (2012) studied fortification of mozzarella cheese using selected sources of omega-3 and non-thermal approaches (PEF and HPP). The growth of coliforms was delayed using thermal pasteurization and pressurization of milk; however, PEF did not delay the growth of coliforms, showing counts around 2.8 log after 8 days of storage. The mesophiles counts of cheeses processed with PEF and thermal treated milk were close to 7 log, while the pressurized milk showed a lower microbial growth of 5.8 log at the end of storage of 12 days. Similar profiles for psychrophiles were observed during storage, with HPP being the most effective treatment for milk processing before cheesemaking. Lopez-Pedemonte, Roig-Sagués, Lamo, Gervilla, and Guamis (2007) demonstrated reductions in *Staphylococcus aureus* counts on HPP treatments (300, 400 and 500 MPa at 5 °C and 20 °C). While reductions obtained for HPP treatments at 5 °C differed significantly between 300, 400 and 500 MPa in both *Staphylococcus aureus* strains, for 20 °C HPP treatments differences only became

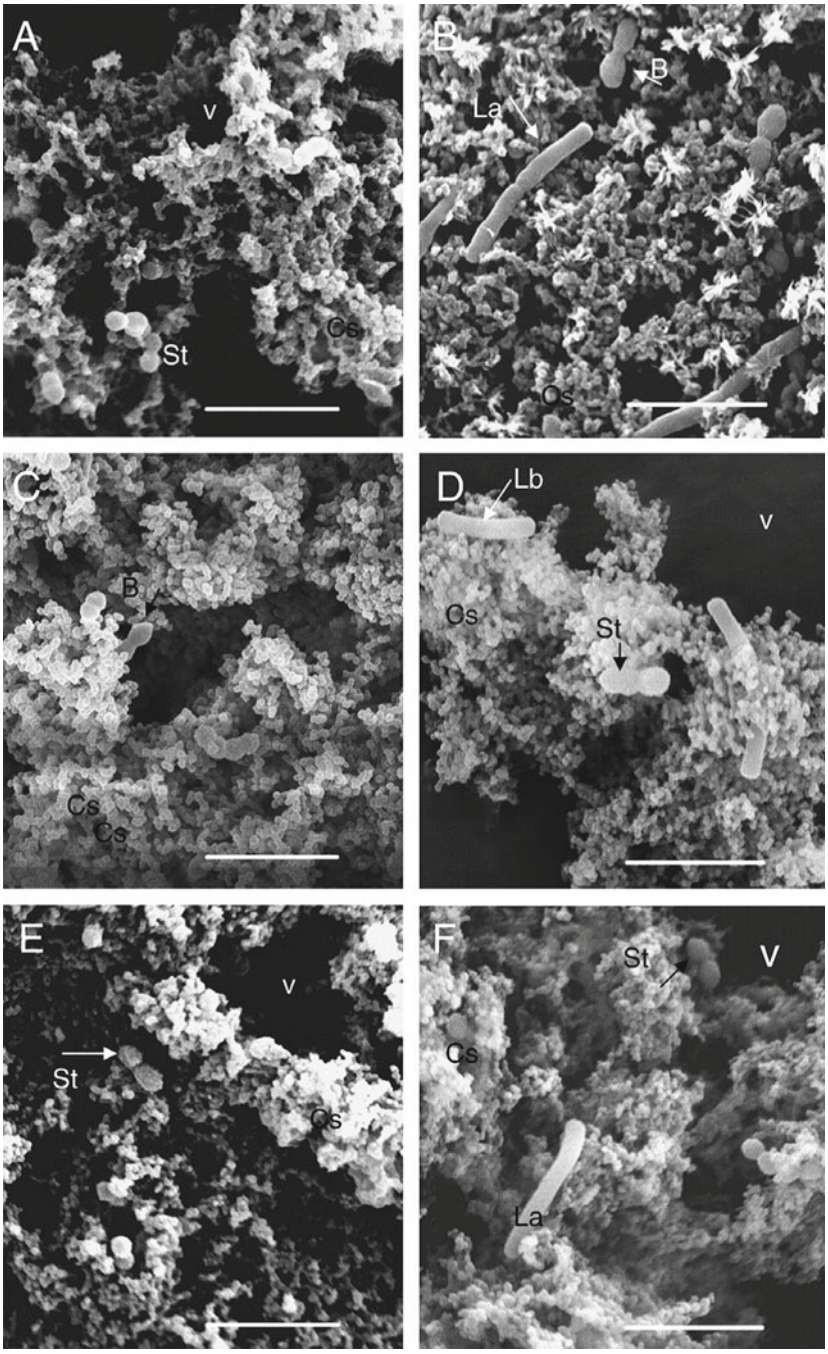

Fig. 4.2 Scanning electron microscopy micrographs of yoghurt fermented with starters YO MIX 236 (**a**, **c**, **e**) and DPL ABY 611 (**b**, **d**, **f**) with different treatments: (**a**, **b**) heat, (**c**, **d**) HPP, (**e**, **f**) heat + HPP. St, *Streptococcus thermophilus*; Lb, *Lactobacillus delbrueckii ssp. bulgaricus;* La, *Lactobacillus acidophilus*; B, *Bifidobacterium longum*; v, void space; cs, casein (From Penna, A.L.B., Subbarao-Gurram, Barbosa-Canovas, G.V. 2007. *Food Research International*.40: 510–519. With permission)

significant after applying 500 MPa pressure. Similarly, Lopez-Pedemonte, Roig-Sagues, Lamo, Hernandez-Herrero, and Guamis (2007) indicated that counts of control samples for both strains of *L. monocytogenes* did not significantly differ during storage. In contrast, counts of all treated samples diminished with storage time.

Serrano, Velazquez, Lopetcharat, Ramirez, and Torres (2005) indicated that short and moderate hydrostatic pressure (MHP) treatments accelerated the shredability of Cheddar cheese. Both MHP (345 MPa for 3 and 7 min) and higher pressure (483 MPa for 3 and 7 min) treatments applied to 1 day milled curd cheddar cheese induced a microstructure resembling that of ripened cheese as shown in Fig. 4.3. The reduction in the amount of crumbles as well as increase in desirable physical properties such as surface smoothness and shred mean length and uniformity in pressure-treated samples was reported. Further, the pH of the control cheese samples increased during storage. During storage, pH fluctuated and the pH difference on day 27 was lower than initial pH difference (day 0) but remained higher for pressure-treated samples with 483 MPa>345 MPa>control. Dhakal et al. (2014) demonstrated that in case of high pressure treatment (450 MPa and 600 MPa) of

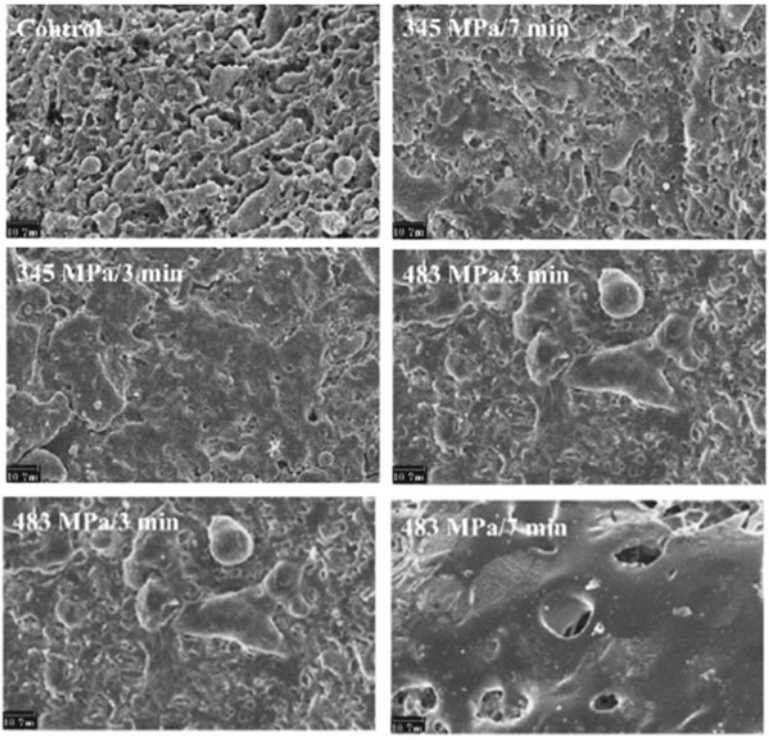

Fig. 4.3 Scanning electron microscope micrographs of control (**a**) and pressure-treated (**b–f**) milled curd cheddar cheese (From Serrano, J., Velazquez, G., Lopetcharat, K., Ramirez, J.A., Torres, J.A. 2005. *J. Food. Sci*. 70 (4): 286–293. With permission)

almond milk, the amandin content, which is the major almond allergen could no longer be detected, whereas, thermally processed samples did not show significant reductions unless the samples were treated at a temperature higher than 85 °C.

4.2.3 Alcoholic Beverages

Currently a range of food products such as fruit juices, sea foods and meat products can be found on market shelves all around the world (Matser, Krebbers, van den Berg, & Bartels, 2004). However, few data have been reported about the use of HPP for beer and wine. Although, rice wine (nigori sake) is one of the earliest HHP-treated commercial products that appeared on the Japanese market (Suzuki, 2002), no HHP-treated alcoholic beverage such as beer and wine is introduced to date.

Wine cannot be treated with heat since its characteristics such as flavour, taste and colour are very sensitive to temperature (Mermelstein, 1998). Therefore, a common practice is the addition of sulphur dioxide (SO_2) to wine to reduce the microbial population of the grape must and to preserve the final product for long period of time (Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2006). SO_2 acts as an antimicrobial agent and antioxidant in wine (Amerine, Berg, & Cruess, 1967; Romano & Suzzi, 1993). However, SO_2 may have negative effects on human health (Romano & Suzzi, 1993). Therefore, wine industry is challenged to meet consumers' demands of reducing the levels of SO_2 used in wine production (Du Toit & Pretorius, 2000). Tabilo-Munizaga et al. (2014) studied the effects of high hydrostatic pressure (HPP 400–500 MPa for 3–10 min) on the protein structure and thermal stability of Sauvignon blanc wine. It was observed that higher thermal stability and major structural changes observed in wine proteins were obtained by 450 MPa pressure for 3 and 5 min. With this pressure–time combination, the structural conformations achieved by the wine proteins could provide higher thermal stability and thus delay haze formation in wine during 60 days storage. Santos et al. (2013) studied the effect of high-pressure treatments on the physico-chemical properties of a sulphur dioxide-free red wine. The wine pressurized at 500 MPa presented more scents of cooked fruit and spicy aroma. The untreated wines presented less perceived fruity and floral aroma and had a more pronounced metallic and leather aroma than the other wines. Comparing the taste assessment of the different wine samples, the pressurized wines presented a similar taste assessment than the wine with SO_2. The untreated wines showed a higher acidity and lower balance. In terms of colour, the pressurized wines presented higher values of brown and limpidity and lower values of violet than unpressurized wines. After 12 months of storage, pressurized wines showed a better sensorial assessment, with the pressure treatments imparting aged-like characteristics to the wines. The results demonstrated that HPP can influence long-term red wine physico-chemical and sensorial characteristics. HPP results in an increase of condensation reactions of phenolic compound. The compounds formed with higher degree of polymerization become insoluble in wine

along storage. Mok et al. (2006) studied the pasteurization of low-alcohol red wine (ethanol 9 %, pH 3.27, acidity 0.068 %, total sugar 0.85 %) using HPP (100–350 MPa for 0–30 min at 25 °C). Corrales, Butz, and Tauscher (2008) applied HPP to wine from the dornfelder grape variety. A decrease in the concentration of malvidin-3-*O*-glucoside in pressurized (600 MPa at 70 °C for 1 h) samples was detected. Wherein, wine subjected to pressure treatment (600 MPa, 70 °C for 10 min) exhibited no significant differences in anthocyanin composition and antioxidant activity.

The aerobic bacteria decreased below the detection limit after 20 min pressurization at 300 MPa and 10 min pressurization at 350 MPa as well as initial yeast count (5.46 log) decreased to 2.46 and 1.15 log after HPP treatment at 300 MPa for 10 and 20 min, respectively. Puig, Vilavella, Daoudi, Guamis, and Minguez (2003) investigated the microbiological and biochemical stabilization of wines by use of HPP. A white wine (with 40–50 mg L^{-1} of total SO_2) and a red wine (with 80–90 mg L^{-1} of total SO_2) were used. Two yeasts: *S. cerevisiae* and *Brettanomyces bruxellensis* (107 CFU L^{-1} of each wine), two lactic acid bacteria: *L. plantarum* and *Oenococcus oeni* (109 CFU L^{-1} of each wine), and two acetic acid bacteria: *A. aceti* and *A. pasteurianus* (109 CFU L^{-1} of each wine) were inoculated into wines. HPP treatments were done at 400 or 500 MPa for 5 or 15 min with 4 °C or 20 °C of temperature. HPP treatments resulted 6 log reduction for yeasts and 8 log reduction for bacteria.

Commercially, beer is pasteurized to guarantee microbiological stability during its shelf life (Zufall & Wackerbauer, 2000). However, heat can cause protein denaturation, promoting the formation of new tannin-protein complexes, with consequent turbidity enhancement (Stewart, 2006). In addition, heat processing promotes the Maillard reaction, resulting in an alteration of beer colour to reddish (Castellari, Arfelli, Riponi, Carpi, & Amati, 2000) and the formation of undesirable flavours (Stewart, 2006). These are related to oxidation and staling, with the development of off-flavours (long chain aldehydes) (Zufall & Wackerbauer, 2000). HPP may be used to increase the shelf life of special quality beers without altering the original characteristics of the untreated product and without heat treatments or filtration. Thus, HPP could be an alternative to the conventional pasteurization of beer. The first trial of HPP on brewing process of beer was carried out by Fischer, Schöberl, Russ, and Meyer-Pittroff (1998). Fischer et al. (1998) concluded that HPP treatment (300–700 MPa, 5 min) of bright lager beer samples packed in polyethylene naphthalate bottles did not significantly change the colour, foam durability and the spectrum of flavour materials. In contrary, Perez-Lamela, Reed, and Simal-Gándara (2004) indicated that HPP treatment (300–600 MPa, 20 min) of beer resulted in an increase in the foaming and haze characteristics of the beer. Gänzle, Ulmer, and Vogel (2001) investigated the effect of ethanol and hop extract on inactivation of *L. plantarum* in model beer system during and after HPP treatment. Addition of ethanol and hop extract accelerated HHP inactivation of *L. plantarum* in model beer. Buckow, Heinz, and Knorr (2005) investigated the combined effect of HPP (0.1–900 MPa) and temperature (30–75 °C) on the activity of β-glucanase from barley malt. Thermostability of β-glucanase was found to be highest at 400 MPa. Wherein, at temperatures above 55 °C and ambient pressure, as well as at (900 MPa, 40 °C), the inactivation of β-glucanase was higher resulting in complete loss of enzyme activity in less than 30 min.

Effect of HPP on quality parameters of lager beer was studied by Buzrul, Alpas, and Bozoglu (2005a). Unpasteurized lager beer samples from a commercial brewery were treated either by HPP (200–350 MPa, 3 and 5 min, 20 °C) or by conventional heat pasteurization (60 °C for 15 min). The colour, protein sensitivity and chill haze values increased as the pressure and pressurization time increased. Change in bitterness was higher in conventional heat pasteurization, but pressures up to 300 MPa had no significant effect on bitterness. In another study Buzrul, Alpas, and Bozoglu (2005b) investigated the effects of HPP on shelf life of lager beer. Filtered bright lager beer samples were either treated with HPP (350 MPa, 3 and 5 min, 20 °C) or by conventional heat pasteurization (60 °C for 15 min). However, HPP-treated samples had lower bitterness and protein sensitivity and higher chill haze values than the heat pasteurized samples at the end of the storage period. Fischer et al. (2006) examined the effect of pH (4.0, 5.0, 6.0 and 6.5) and HPP treatment (300 MPa, 20 °C for various holding times up to 120 min) on *L. plantarum* (moderately hop tolerant) in model beer system. It was observed that inactivation was more effective at lower pH values, where up to 5 log reductions could be achieved.

4.2.4 Bakery Products

Different scientific reports described the effect of HPP on specific cereal components properties or model systems, namely starch and gluten (Apichartsrangkoon et al., 1998; Gomes et al., 1998; Kieffer et al., 2007). HPP induced starch gelatinization, following different mechanism than thermally induced gelatinization (Gomes et al., 1998). HPP treatment provokes swelling of starch but keeping granule integrity; as a consequence HPP-treated starches modify their microstructure and rheological properties in a different way than thermally treated ones (Gomes et al., 1998; Stolt et al., 2000).

Scanning electron microscopy was used to determine the effect of the HPP on dough microstructure. Scanning electron micrographs of wheat doughs treated at 50, 150 and 250 MPa for 4 min are showed in Fig. 4.4. Untreated wheat dough was characterized by having a continuous structure with the intact starch granules embedded in the matrix structure of proteins and soluble solutes. Dough treated at pressure of 50 and 150 MPa showed well-defined starch granules with diverse size, and the surrounding structures (mainly of protein nature) were progressively reduced, being confined in the case of 150 MPa to agglomerates of starch granules. Drastic changes were observed in dough treated at 250 MPa where starch granules as individual structures disappeared adopting a discontinuous film-like organization similar to what happened after swelling and gelatinization (Barcenas, Altamirano-Fortoul, & Rosell, 2010). Barcenas et al. (2010) reported that the treatment of wheat dough with HPP induced a rapid reduction of the microbial population but sufficient mould and yeast survival, for ensuring bread dough fermentation at moderate pressure conditions (50–250 MPa, for 2 min at 20 °C). This study suggests that high hydrostatic processing in the range 50–200 MPa could be an alternative technique for obtaining novel textured cereal based products.

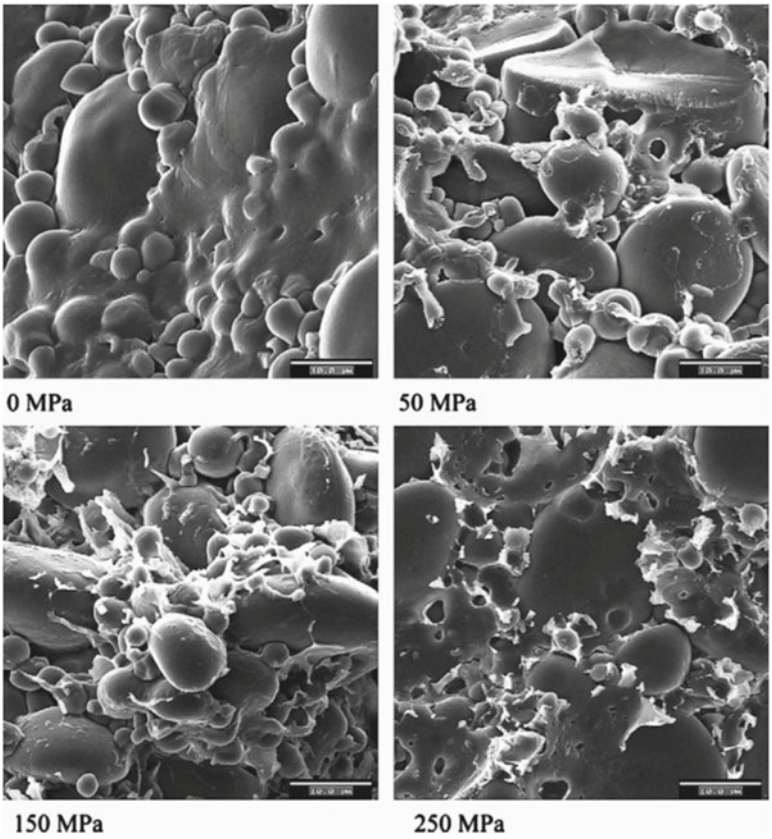

Fig. 4.4 Scanning electron micrographs of wheat dough exposed to varying pressure levels (0–250 MPa/4 min) (From Barcenas, M.E., Altamirano-Fortoul, R., Rosell, C.M. 2010. *LWT—Food Science and Technology*.43: 12–19. With permission)

4.3 Concluding Remarks and Future Outlook

HPP has attracted considerable interest in food industry due to its promising effects in food preservation. Consumers require safe and natural products without added preservatives. Consuming fermented foods such as probiotic drink (kefir, kombucha), sauerkraut, kimchi, as well as fermented meat products helps in maintaining proper balance of gut bacteria and digestive enzymes. Synergistic effect of HPP in combination with multiple hurdles such as low pH, a_w and acidic environment in fermented foods helps in achieving an increased rate of inactivation of spoilage microbes and endogenous enzymes. The hygienic quality of raw materials may be improved by decreasing microbial load through pasteurization, in the dairy industry. However, conventional heat treatments in the case of fermented meat products cause

detrimental changes. Hence, alternative mild preservation techniques like high pressure processing show promising opportunities. However, there are certain technological limitations. For example, bacterial spores are highly pressure resistant, since pressures exceeding 1200 MPa may be needed for their inactivation (Knorr, 1995). Sterilization of low-acid foods (pH > 4.6) requires combination of high pressure and other forms of relatively mild treatments like heating (Rastogi & Raghavarao, 2007). Nonetheless, HPP has promising applications to satisfy consumer demand for high-quality food products. HPP enables extended shelf life and safety of fermented meat and dairy products with improved sensory and organoleptic characteristics. Hence, HPP can be considered as an innovative technique that can be employed by the food industry to meet the raising consumer demands for safe and nutritious fermented food products.

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Chapter 5
Pulsed Electric Field and Fermentation

T. Garde-Cerdán, M. Arias, O. Martín-Belloso, and C. Ancín-Azpilicueta

5.1 Introduction

Pulsed electric field (PEF) is a fast and effective technology to induce the electroporation and permeabilization of cell membranes. It has been widely employed in molecular biology, biotechnology, and genetic engineering to introduce genes or plasmid DNA in living cells (Escoffre et al., 2009). The capacity of pulsed electric fields for promoting cell poration and enzyme deactivation has drawn the attention of the food sector, to the extent that PEF treatments are currently evaluated to be employed as a treatment for the preservation of fluid foods. By applying PEF, it is possible to promote the formation of irreversible porous in the cell membranes of microorganism present in food. The irreversible poration of cell membranes causes a loss of the homeostasis and subsequent cell death (Saulis, 2010). Endogenous enzymes that trigger food oxidation processes are also inactivated towards PEF

T. Garde-Cerdán
The Vine and Wine Science Institute (La Rioja Regional Government –CSIC – La Rioja University), Carretera de Burgos, Km. 626007, Logroño, Spain
e-mail: Teresa.GardeCerdan@gmail.com

M. Arias
Institute for Global Food Security (IGFS), School of Biological Sciences, Queen's University Belfast, Belfast, Northern Ireland, UK
e-mail: margaluz.arias@gmail.com

O. Martín-Belloso
CeRTA-UTPV, Food Technology Department, University of Lleida,
Avda. Alcalde Rovira Roure, 191, 25198 Lleida, Spain
e-mail: omartin@tecal.udl.es

C. Ancín-Azpilicueta (✉)
Applied Chemistry Department, Public University of Navarre,
Campus Arrosadía s/n, 31006 Pamplona, Spain
e-mail: ancin@unavarra.es

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treatments (Vega-Mercado et al., 1997). In addition, by applying PEF, other molecules contained in the food are not modified in most cases (Vega-Mercado et al., 1997). This means that PEF has an advantage over other traditional preservation treatments based on thermal processes where part of nutrients are partially depleted or structurally modified to form toxic compounds (Markowicz-Bastos, Monaro, Siguemoto, & Séfora, 2012).

The inactivation of spoilage microorganism such as moulds, yeasts, and bacteria present in fruits and vegetable juices, using PEF treatments, has been appraised by several research groups. Thus, PEF treatments have been applied in orange juice (Elez-Martínez, Escolà, Soliva-Fortuny, & Martín-Belloso, 2005; Elez-Martínez, Escolà-Hernández, Soliva-Fortuny, & Martín-Belloso, 2004; McDonald, Lloyd, Vitale, Petersson, & Innings, 2000); apple juice and cranberry juice (Sen Gupta, Masterson, & Magee, 2003); orange, apple and watermelon juices (Timmermans et al., 2014); orange-carrot juice and broth (Selma, Salmerón, Malerov, & Fernández, 2004); fruit-juice milk beverages (Salvia-Trujillo, Morales-de la Peña, Rojas-Graü, & Martín-Belloso, 2011); melon and watermelon juices (Mosqueda-Melgar, Raybaudi-Massilia, & Martin-Belloso, 2007), among others.

The efficiency of PEF for microorganism deactivation strongly depends on the microorganism strain and specie, the media, and PEF process conditions. Maximum reductions observed in bacterial and yeast populations after PEF treatments are in a range of 3–6 log units (Elez-Martínez et al., 2004; Mosqueda-Melgar, Elez-Martínez, Raybaudi-Massilia, & Martín-Belloso, 2008). It has been observed that yeast are more PEF-sensitive than bacteria (Marsellés-Fontanet, Puig, Olmos, Mínguez-Sanz, & Martín-Belloso, 2009; Mosqueda-Melgar et al., 2007; Toepfl, Heinz, & Knorr, 2007). At the same time, it has been observed that microorganism inactivation by PEF is more efficient when treatments are applied to acidic media or media with low values of conductivity (Raso, Calderón, Góngora, Barbosa-Cánovas, & Swanson, 1998; Timmermans et al., 2014).

In most of the studies carried out, it has been observed that PEF treatments did not cause important variations on the physicochemical properties of juices subjected to the treatment. This means that, in general, the PEF-treated products retained their original organoleptic and nutritive properties (Garde-Cerdán, Arias-Gil, Marsellés-Fontanet, Ancín-Azpilicueta, & Martín-Belloso, 2007; Guo et al., 2014; Marsellés-Fontanet, Puig-Pujol, Olmos, Mínguez-Sanz, & Martín-Belloso, 2013; Vega-Mercado et al., 1997). So, the capability of PEF to deactivate spoilage microorganism without detrimental effects on the quality of the product means that this technique could be of potential use in winemaking.

Wine is the resulting product of grape juice fermentation. The alcoholic fermentation is carried out by yeast, mainly yeast of the genus *S. cerevisiae*. These yeasts are naturally present on the grape skin and bunch. They also can be added to the must at the beginning of the alcoholic fermentative to speed up the fermentation, to obtain a controlled and homogeneous product, or to improve the fermentative flavours of the final wine (Lambrechts & Pretorious, 2000). The natural microbiota of grape skins also includes spoilage microorganisms which are prone to development during the fermentative and ageing processes. The yeast of genus *Brettanomyces*/*Dekkera* and acetic acid bacteria are the most damage-causing spoilage microorganism present in

grape skin and on winery surfaces (Cocolin, Rantsiou, Iacumin, Zironi, & Comi, 2004). *Brettanomyces*/*Dekkera* metabolizes hydroxycinnamic acids of the grape into ethylphenols. Those compounds, including 4-ethylphenol and 4-ethylguayacol, are responsible for odours of "leather", "animal", and "horse sweat" (Garde-Cerdán et al., 2010; Garde-Cerdán, Rodríguez-Mozaz, & Ancín-Azpilicueta, 2002). These odours are unpleasant and consequently decrease the organoleptic characteristics of the resulting wine. *Brettanomyces*/*Dekkera* mostly develops in wine barrels as a result of improper cleaning practices (Suárez, Suárez-Lepe, Morata, & Calderón, 2007).

Spoilage bacteria of the genus lactobacillus, such as *Lactobacillus hilgardii*, can use residual sugars which remain in the media due to slow alcoholic fermentations. The bacteria transform the sugars into lactic and acetic acids increasing in this manner the volatile acidity of the final product which causes the "pique lactique" (Lonvaud-Funel, 1999). The development of those spoilage microorganisms during fermentation and wine ageing causes several problems for the wine industry and could lead to important economic losses (Loureiro & Malfeito-Ferreira, 2003; Snowdon, Bowyer, Grbin, & Bowyer, 2006).

The addition of SO_2 in winemaking prevents and controls the development of those spoilage microorganisms in must and wine. SO_2 prevents wine spoilage, but also naturally avoids occurring oxidative and enzymatic reactions. These reactions, if not avoided, may lead to detrimental modifications on the colour and taste of the final wine (Lustrato et al., 2003). However, sulphites derived from the addition of SO_2 cause allergic reactions to the asthmatic population (Timbo, Koehler, Wolyniak, & Klontz, 2004; Vally & Thompson, 2003). SO_2 also causes the simultaneous activation of onco-genes and the inactivation of tumour suppression genes in people with severe hypersensitivity to this molecule or in patients under continuous steroid treatments. Hence, these effects have been related to the SO_2-linked lung cancer (Qin & Meng, 2009).

With this point in mind, the International Organisation of Vine and Wine (OIV) has reduced the authorized content of SO_2 in wines (Regulation (EC) No. 607/2009) and it is expected to be further reduced in the coming years. Thus, an alternative treatment process to avoid the proliferation of spoilage microorganism, which provides the same advantages as SO_2 but without its linked health risks, is urgently needed.

The application of pulsed electric fields to must seems to be one of the most promising treatments in substitution of SO_2 (Santos, Nunes, Saraiva, & Coimbra, 2012). The process has been proven to effectively reduce microorganism population without affecting the original properties of the product (Donsì, Ferrari, & Pataro, 2010; Garde-Cerdán, Arias-Gil, et al., 2007; Marsellés-Fontanet et al., 2013; Vega-Mercado et al., 1997). Furthermore, the treatment has been shown to inactivate natural-occurring enzymes, responsible for detrimental organoleptic modifications, in a similar manner as SO_2 (Marsellés-Fontanet & Martín-Belloso, 2007). The enzymes polyphenol oxidase (PPO) and peroxidase (POD) are present in grape must and catalyse the oxidative reactions of polyphenols to form brown polymeric pigments (Li, Guo, & Wang, 2008; Singleton, 1987). These enzymatic reactions, if not controlled, can confer a detrimental brown colour to wines (Li et al., 2008).

Another application of PEF treatments in winemaking is closely related with the potential of this technology to promote mass transfer processes of vegetable cells (Donsì, Ferrari & Pataro, 2010). In red wine production, the alcoholic fermentation of the must is carried out in the presence of grape skins, and this procedure is called maceration-fermentation process. This practice is aimed at obtaining maximum extraction of phenolic compounds from grape skins. Phenolic compounds contribute to the organoleptic characteristics, age ability, and health attributes of red wines. Thus, anthocyanins, which are involved in the formation of polymeric pigments, play an important role on colour stabilization of wines during ageing (Guadalupe & Ayestarán, 2008). Proanthocyanidins also contribute to the colour as well as to the astringency and bitterness of the wine (Del Llaudy, Canals, Canals, & Zamora, 2008). Proanthocyanidins and catechins, due to their antioxidant and radical-scavenging properties, are associated with the health benefits of moderate consumption of wine (Nichenametla, Tarusicio, Barney, & Exon, 2006; Rice-Evans, Miller, & Paganga, 1996). The application of PEF to crushed grapes has been shown to increase the permeability of grape cells (Cholet et al., 2014; Delsart et al., 2014). Therefore, the treatment improves, in a significant manner, the extraction of phenols and other beneficial compounds from grape skins during the maceration-fermentation process (Garde-Cerdán et al., 2013; López-Alfaro et al., 2013; López-Giral et al., 2015; Puértolas, López, Condón, Álvarez, & Raso, 2010a, 2010b). This leads to the production of high-quality wines through short maceration or maceration-fermentation periods (Luengo, Franco, Ballesteros, Álvarez, & Raso, 2014; Puértolas, Saldaña, Álvarez, & Raso, 2011).

This chapter provides a brief introduction to PEF technologies and its fundamentals. It also summarizes the potential applications of PEF treatments to improve the alcoholic fermentation of must, through the different studies that have been performed in this respect. These include the application of PEF to inactivate the microbial population of grape must and its potential as an alternative method to reduce or eliminate the addition of SO_2 during the alcoholic fermentative process and ageing in bottles. It comprises, as well, the potential uses of PEF to increase the rate and the yield of mass transfer processes occurring during the vinification of red and rosé wines. In addition, the benefits of PEF on aiding the pressing process of grapes for white winemaking are mentioned.

5.2 PEF Technology

Pulsed electric field technology is based on the application of electric pulses of high strength applied to fluid samples through two electrodes of opposite charge situated inside a treatment chamber where an electric field is created. The electric pulses applied are of short duration, in the order of μs and can be of different wave shapes. Pulse waves can be rectangular, of exponential decay, sinusoidal, modulatory, or of instantaneous inverse charge. However, the most commonly employed for the treatment of food commodities are of rectangular wave and of exponential decay. The applied electric field strength of rectangular pulses is maintained during the

duration of the pulse. This wave is produced with special switches that are able to stop the current at high voltages. The electric field strength (E, kV/cm) depends on the potential (V, kV) applied within the two electrodes and the distance between the electrodes (d, cm).

$$E = V / d \tag{5.1}$$

The electric strength of the pulse determines the efficiency of the treatment. Other important parameters are the number of pulses (n), their frequency (F, Hz), and the treatment time (t_t, ms). The treatment time is the product of the pulse width and the number of pulses. In the case of continuous flow treatment, the number of pulses is the result of the frequency and the time of residence of the fluid food in the treatment chamber.

The relative energy (W, MJ/kg) employed in a treatment is calculated as the product of the electric field strength (E, kV/cm), the conductivity of the sample (α, S/m), and the time of treatment (t_t, ms) (Marsellés-Fontanet et al., 2009).

$$W = E \times \alpha \times t_t \tag{5.2}$$

In exponential decay pulses, an initial electric strength is applied and immediately after, the capacitor is led to discharge. The decay time is the duration of the pulse and it can be calculated as the product of the capacitance and sample resistance or sample conductivity (Jordan, Collins, Terefe, Ugozzoli, & Rubio, 2008). The energy involved in a single pulse is defined by:

$$W^1 = 1 \Big/ \rho \int_0^\infty I(t).V.d(t) \tag{5.3}$$

where W is the relative energy (MJ/kg), I is the current intensity (A), V is the voltage applied (kV), t is the pulse decay duration (μs), and ρ is the number of pulses. The total energy is the result of the pulse number and the relative energy.

PEF treatment can be applied in a batch mode by maintaining the sample inside the chamber during the treatment. It can also be applied in a continuous flow passing the fluid food through the treatment chamber by a peristaltic pump. The chambers can be situated in series or in parallel and the product can be re-circulated throughout the chambers to ensure that sample has been treated homogeneously (Elez-Martínez et al., 2005). The devices are equipped with a cooling system that maintains the temperature of the sample under control to avoid overheating derived from the electric process.

The treatment with pulsed electric fields (PEF) induces a series of mechanical transformations in membranes of eukaryotic and prokaryotic cells. Cell membranes behave as a capacitor of low dielectric constant. When cells are exposed to an external electric field of high potential, ions of opposite charge are distributed at both sides of the membrane. In turn, an inter-membrane electric field is created. These charges attract each other causing the compression of the lipid membrane in certain points. Depending on the electric field applied and the number of pulses, a critical trans-membrane potential is reached. Pulse treatments that induce inter-membrane potentials close to the critical values promote the

formation of reversible pores in the membrane. However, irreversible pores are formed when the inter-membrane critical potential is reached or overwhelmed. This causes important morphological changes and the disintegration of the cell membranes (Donsì, Ferrari & Pataro, 2010; Heinz, Álvarez, Angersbach, & Knorr, 2002; Toepfl et al., 2007).

The critical inter-membrane potential is also related to the cell size, in a manner that, the lower the cell diameter is, the higher the critical inter-membrane potential is. In consequence, higher electric field strength had to be applied to infer an effect in membranes of small size cells compared with cells of higher dimensions (Álvarez, Condón, & Raso, 2006; Hülsheger, Potel, & Niemann, 1983; Zimmermann, Pilwat, & Riemann, 1974). Furthermore, the critical inter-membrane potential is also a function of the shape of the cell. Induced trans-membrane potentials for spherical cells are related to the ratio of the cell and the radial direction vector of the applied electric field. For ellipsoidal cells, the critical trans-membrane potential value is determined by the distance between the surface and the centre of the cell in the direction of the electric field applied (Heinz et al., 2002). The length of the cell, the diameter, and the outer base determined the trans-membrane potential in rod-shaped cells. Thus, the influence of the microorganism shape is such that the induced inter-membrane potentials required for irreversible poration of road-shaped cells are five times higher than for ellipsoidal cells, when both cells have the same dimensions (Heinz et al., 2002).

In addition to the effect that pulsed electric fields have on cells membranes, it has also been observed that the treatment inactivates enzymes contained in the samples (Vega-Mercado et al., 1997). It has been suggested that enzyme inactivation is caused by the disruption of the secondary structure. The reduction of the α-helix fractions and subsequent decrease of residual enzyme activity have been observed as a result of PEF treatments on enzymatic solutions (Zhong et al., 2007). The enzymatic inactivation induced by PEF increases with the increment of electric field strength, the frequency, and the treatment times (Zhao, Yang, Lu, Tang, & Zhang, 2007). The conductivity of the media is also implicated in the enzyme inactivation inferred by PEF. Therefore, the treatments inactivate enzymes more efficiently when they are dissolved in food commodities than in low ionic strength buffers (Yang, Li, & Zhang, 2004). The structural stability of enzymes is closely related to a balance of non-covalent interactions, mainly electrostatic (Koumanov, Ladenstein, & Karshikoff, 2001). Electrostatic interactions depend on the pH and the ionic strength of the product as well as the electric field applied. Thus, under an electric field of high strength, the electrostatic interactions within the protein structure can be disrupted. This effect may be intensified at high ionic strengths (Koumanov et al., 2001; Yang et al., 2004).

5.3 PEF Treatments Applied to Must Preservation

5.3.1 Sterilization of Must

Initial studies to asses PEF effects on grape-juice spoilage microorganisms have been performed by applying the treatment to inactivate yeast and bacteria suspended in water or model solutions. In this manner, the effect of PEF parameters is

evaluated in function of the target microorganisms in a controlled environment (Hülsheger et al. 1983; Vega-Mercado, Pothakamury, Chang, Barbosa-Cánovas, & Swanson, 1996). In a study carried out by Geveke and Kozempel (2003), it was shown that *S. cerevisiae* and *Candida stellata* suspended in water were reduced more than 3 log units with exponential decay pulses at 30 °C with an applied electric strength of 12.5 kV/cm and 20 pulses of 0.3 ms duration. Bacteria included in this study, since they are of a smaller size than yeast, were more resistant to the treatment. Thus, *Escherichia coli* and *Listeria innocua* were only reduced by 1 log unit on applying the same treatment conditions (Geveke & Kozempel, 2003). The application of the same treatment in acidified media effectively reduced *L. innocua* by 3 log units, while the inactivation of *E. coli* under acidic environment remained at the same level as in neutral media (Geveke & Kozempel, 2003). The higher effect of PEF for the inactivation of microorganism in acid media respect of neutral media has been also observed by other authors (Timmermans et al., 2014).

Our research group investigated the effect of PEF parameters on the inactivation of *Kloeckera apiculata*, *Saccharomyces cerevisiae*, *Lactobacillus plantarum*, *Lactobacillus. Hilgardii,* and *Gluconobacter oxydans* inoculated on Parellada must (Marsellés-Fontanet et al., 2009). A continuous flow PEF equipment was employed (Fig. 5.1). Treatment chambers of 12 mm^3 volume and 2.9 mm electrode-gap distance were positioned in series to allow maximum treatment efficiency. The must was maintained at temperatures below 35 °C by connecting cooling coils between each pair of treatment chambers. The electric pulses, of square wave shape and bipolar mode, were applied at a fixed width of 5 μs. The applied electric field strength, frequency, and treatment times were modified to achieve maximum microorganism inactivation. By applying statistic models to the observed results, it was calculated that maximum reductions of 4 log units were obtained for *K. apiculata*, *S. cerevisiae,* and lactic acid bacteria by applying electric field strengths in a range

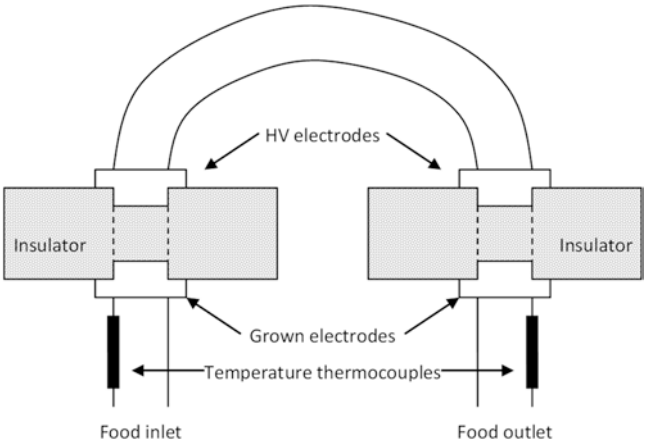

Fig. 5.1 Scheme of a pair of treatment chambers of the PEF equipment used

of 24.9–35 kV/cm, frequencies of 100–333 Hz, and treatment times between 0.87 and 1 ms. The electric field at the values applied in this study did not influence the inactivation of the selected yeast. However, the inactivation of *K. apiculata* and *S. cerevisiae* was mostly affected by the frequency and the treatment times. The frequency influences the efficiency of PEF treatments, since it allows the modulation in which the energy is applied to the sample (Bolado-Rodríguez, Góngora-Nieto, Pothakamury, Barbosa-Cánovas, & Swanson, 2000; Marsellés-Fontanet et al., 2009). The bacteria studied were also sensitive to high electric fields, being more resistant than yeast with the treatment. Thus, although the lactic acid bacteria were inactivated at the same level as yeast, this was achieved by applying more drastic conditions. Furthermore, only 1.1 log unit reduction was obtained for *G. oxydans,* applying 27.5 kV/cm, 600 Hz, and 1 ms of treatment.

The lethal effect of PEF in grape must inoculated with *S. cerevisiae* and pathogenic bacteria (*E. coli* DH5 and *S. aureus* was assessed by Huang, Yu, Wang, Gai, and Wang (2014)). At temperatures ranging from 40 to 49 °C, by using monopolar square wave pulses of 3 μs and a frequency of 120 Hz, *S. cerevisiae* population was reduced by more than 6 log units with electric field strengths of 27 kV/cm for 45 μs and also with 24 kV/cm for 68 μs. However, *E. coli* DH5 and *S. aureus* were reduced by 2.2 and 3.3 log units, respectively, with field intensities of 27 kV/cm and treatment times of 275 μs.

Puértolas, López, Condón, Raso, and Álvarez (2009) examined the effect of PEF on the inactivation of yeast and bacteria inoculated in musts and wines. Pulsed electric fields of exponential decay and fixed frequency of 1 Hz were applied to the inoculated samples at different electric field strength and relative energies. The yeasts studied, *Dekkera anomala*, *Dekkera bruxellensis,* and *Saccharomyces bayanus*, were affected by the treatment at field intensities above 16 kV/cm, while inactivation of bacteria, *Lactobacillus hilgardii* and *Lactobacillus plantarum*, was reached at field intensities above 20 kV/cm. Thus, maximum inactivation of 3 log units reductions of the microorganisms was achieved at electric field strengths in the range of 29–31 kV/cm and respective specific energies in a range of 396–194 kJ/kg. Under the same PEF treatment conditions, inactivation of the yeasts and *L. hilgardii* was higher in wines than in musts. The diffusion of rich ethanol media through the porous of sublethal-PEF-injured cells may inactivate important microbial enzymes. This could cause the failure and subsequent death of the cell (Heinz & Knorr, 2000). It was also suggested that polyphenols contained in red wines may contribute to microorganism inactivation, since these compounds have antiseptic properties and may penetrate into the cytoplasm of PEF-injured cells (Campos, Couto, & Hogg, 2003; Stead, 1995).

Different response to the treatment was observed for *L. plantarum* as it was more affected by the treatment in must than in wines (Puértolas et al., 2009). This was explained by the authors as a consequence of the high resistance of these bacteria to high ethanol environments. In this study, it was also observed that the treatment did not affect in any significant manner the colour and aroma of the musts and wines (measured by sensorial analysis), even after the application of the highest electric field intensities.

5.3.2 Inactivation of Must Oxidative Enzymes

Apart from the sterilizing properties of the treatment, PEF processes have the capability to inactivate oxidative enzymes contained in vegetable and fruit juices. The inactivation of enzymes that cause the degradation of must and wines is attained by the addition of SO_2. Thus, the potential of PEF treatments to substitute SO_2 also covers this scope. Wine-browning phenomenon is mainly caused by oxidative enzymes, such as polyphenol oxidase (PPO) and peroxidase (POD), which are naturally occurring in grape must and other fruit juices. Polyphenols of grape musts are oxidized by these enzymes to form quinones (Singleton, 1987). These quinones polymerized to form brown polymeric pigments that imply a detrimental effect on wine colour (Li et al., 2008). Anthocyanins contained in red wines undergo condensation with tannins to form polymeric pigments that stabilize the colour of the final wine (Mayén, Barón, Mérida, & Medina, 1997). Thus, the browning process is mostly related to white musts and wines, since they do not contain anthocyanins, hence they are more exposed to oxidative browning processes than the red wines (Mayén et al., 1997).

Our research group examined the application of PEF to Parellada must (white must) for the inactivation of PPO and POD enzymes. PEF treatments were applied in continuous mode with square wave pulses. Electric field strength, frequency, and treatment times were optimized for maximum enzyme inactivation at a fixed pulse width of 4 μs. Then, the effect of pulse width was examined at the parameters previously optimized. Results revealed that all the parameters tested had an influence on the inactivation of the enzymes. Increased values of electric field strength, frequency, and treatment times implied a decrease of enzymatic activity. The pulse width also had an influence on the enzymatic inactivation, but the trend was not clear (Marsellés-Fontanet & Martín-Belloso, 2007). It was also observed that PPO was more sensitive to PEF treatments than POD. According to the predictive model, PPO could be totally inactivated by applying electric field strength of 35 kV/cm, 5 ms of treatment time, and 630 Hz of frequency. However, maximum inactivation of 50 % could be achieved for POD applying the same treatment time of 5 ms and 30.2 kV/cm of electric strength, and 100 Hz of frequency (Marsellés-Fontanet & Martín-Belloso, 2007). Although POD is also involved in white wines browning, the implication of POD is lower than PPO. Furthermore, the degradation of polyphenols by POD only occurs in the presence of PPO (Li et al., 2008). Therefore, PEF treatments can be positively used to avoid the browning process of white wines. Furthermore, a strong correlation between the electric field strength and pulse frequency was found for the inactivation of both enzymes, meaning that both parameters have to be considered for the inactivation of PPO and POD enzymes by PEF (Marsellés-Fontanet & Martín-Belloso, 2007).

5.4 Application of PEF Technology in Wine Production

5.4.1 Reduction of SO_2 Addition

The application of PEF, as previously mentioned, reduces the microbial population and naturally inactivates occurring enzymes which trigger oxidative reactions. These effects may permit the vinification of must in the absence of or with reduced amounts of SO_2. Our investigation group carried out a research project intended to evaluate the fermentation of white must in the absence of or with a reduced concentration of SO_2. The study included: (1) the evaluation of PEF effects on the physicochemical parameters of initial must and final wines as well as on the concentration of nutrients including fatty acids and free amino acids; (2) the evolution of the concentration of free amino acids, biogenic amines, and volatile compounds during the vinification processes carried out with and without SO_2; (3) the evolution of the volatile composition of resulting wines during ageing in bottle, at two different temperatures, without the addition of SO_2; (4) alternative treatment to reduce the concentration of SO_2 during the vinification.

5.4.1.1 Enological Parameters, Fatty Acids, and Amino Acids

It is necessary to evaluate to what extent PEF treatments affect the original properties of the juice intended for wine production as well as the kinetic of the fermentation. It was observed that the treatment of Parellada must, applied at the optimized PEF parameters, did not affect the enological parameters of this product. Thus, there were no significant changes in the reducing sugar content, pH, and total acidity of PEF-treated must in comparison with non-treated must (Garde-Cerdán, Arias-Gil, et al., 2007).

To characterize the kinetics, the process rate has been calculated from fermentation curves, as an average percentage of the daily-consumed sugar in the ranges of 5–50 % (vf5-50) and 0–99 % (vf0-99) of total sugars (Houtman & du Plessis, 1985). A daily consumption of sugars during the inoculated fermentation with SO_2 was somewhat lower than in the inoculated fermentations without SO_2 (Garde-Cerdán, Marsellés-Fontanet, Arias-Gil, Martín-Belloso, & Ancín-Azpilicueta, 2007), probably because the SO_2 inactivated part of the inoculated yeasts population.

The wines obtained from the inoculated must fermented with or without SO_2 did not show the presence of acetic acid (Table 5.1). This result could be due to the fact that the inoculated yeast (*S. cerevisiae* Na33 strain) produces a very low concentration of acetic acid (Garde-Cerdán & Ancín-Azpilicueta, 2006). The concentration of acetaldehyde was higher at the beginning of the fermentation in the samples fermented in presence of SO_2 than in those fermented without SO_2, which is known to enhance the production of acetaldehyde (Herraiz, Martín-Álvarez, Reglero, Herraiz, & Cabezudo, 1989). The addition of SO_2 induced significantly higher acetaldehyde production by yeast because this molecule binds SO_2 and, in this form, the toxicity of SO_2 is reduced. At the end of the fermentation, higher concentration of acetalde-

Table 5.1 Oenological parameters at 25, 50, and 75 % of consumed sugars and of the final wines obtained from fermentation with SO_2 (20 mg/L) and without SO_2 of Parellada samples treated by PEF

Sample	pH	Acetic acid (g/L)	Total acidity (g/L)[a]	Total SO_2 (mg/L)	Acetaldehyde (mg/L)	Total polyphenols (mg/L)	Alcohol (v/v %)
25 % of consumed sugars							
With SO_2	3.19 ± 0.04 a	–	3.48 ± 0.09 a	10 ± 0	28 ± 1 a	243 ± 3 a	2.2 ± 0.2 a
Without SO_2	3.17 ± 0.03 a	–	4.67 ± 0.04 b	–	7.3 ± 0.5 b	192 ± 4 b	2.1 ± 0.2 a
50 % of consumed sugars							
With SO_2	3.22 ± 0.04 a	–	3.5 ± 0.2 a	13 ± 1	17.5 ± 0.2 a	203 ± 4 a	4.2 ± 0.2 a
Without SO_2	3.17 ± 0.02 a	–	4.1 ± 0.1 b	–	11 ± 1 b	189.5 ± 0.6 b	4.1 ± 0.1 a
75 % of consumed sugars							
With SO_2	3.20 ± 0.04 a	–	3.88 ± 0.06 a	10.8 ± 0.5	9.8 ± 0.5 a	180 ± 8 a	6.3 ± 0.5 a
Without SO_2	3.17 ± 0.03 a	–	4.21 ± 0.06 b	–	12 ± 1 b	190.5 ± 0.6 a	6.1 ± 0.1 a
Wine							
With SO_2	3.24 ± 0.02 a	–	4.1 ± 0.1 a	8 ± 1	1.7 ± 0.5 a	122 ± 2 a	8.3 ± 0.2 a
Without SO_2	3.25 ± 0.01 a	–	4.33 ± 0.07 a	–	8 ± 1 b	122 ± 4 a	8.5 ± 0.5 a

All parameters are given with their standard deviation ($n = 4$)

[a]Expressed as g/L tartaric acid. Different letters indicate significant differences between the samples with and without SO_2 ($p < 0.05$) (From Garde-Cerdán, Marsellés-Fontanet, et al., 2007)

hyde was observed in the wine obtained from the fermentation without SO_2. During the alcoholic fermentation, the concentration of total polyphenols decreased. Polyphenols (including catechins, proanthocyanidins, cinnamic acids, and their derivatives) are subject to oxidation so that the initial yellow straw colour of white wines turns into the deep golden yellow typical of browned wines (Margheri, Tonon, & Trepin, 1980). The wines obtained from both fermentations, with or without SO_2, presented similar levels of total polyphenols (Table 5.1), therefore the SO_2 content did not affect the final polyphenol concentration.

Fatty acids and sterols have a great influence on the growth of fermentative yeast, and thus, on the development of alcoholic fermentation, as they have a remarkable influence on the transport of amino acids through the microbial membrane and the activity of the membrane-linked enzymes such as ATPase. Moreover, the lack of unsaturated fatty acids decreases the yeast tolerance to ethanol. Must nitrogen compounds are also essential in the metabolism of yeasts because nitrogen is, after carbon, the second element utilized during their growth. The content of nitrogen compounds also affects the kinetics of fermentation. In addition, amino acids participate in aroma development and health-related metabolic by-products.

The results showed that the total fatty acid content in the PEF-treated grape juice was slightly lower than in the control must (Table 5.2). These values suggest that the processing treatment has little effect on total fatty acid content. The PEF treatment caused a reduction in the concentration of lauric acid in the grape juice (Table 5.2). This result is in agreement with those obtained by Zulueta, Esteve, Frasquet, and Frígola (2007). Probably, the degasification system of the PEF equipment could cause a little release of C12, due to the volatile characteristic of this molecule. On the other hand, linolenic and oleic acid contents were unaffected by the treatment. These are the most necessary fatty acids during the normal growth of yeasts. In addition, both fatty acids act as survival factors at the end of fermentation

Table 5.2 Concentration of fatty acids (mg/L) measured on fresh and processed *Parellada* grape juice

Fatty acid	Control	PEF
Caprilic acid (C8)	–	–
Capric acid (C10)	–	–
Lauric acid (C12)	0.32 ± 0.01 a	0.19 ± 0.03 b
Myristic acid (C14)	0.37 ± 0.05 a	0.38 ± 0.03 a
Palmitic acid (C16)	14.55 ± 0.08 a	13.8 ± 0.4 a
Palmitoleic acid (C16:1)	–	–
Stearic acid (C18)	1.07 ± 0.01 a	1.11 ± 0.09 a
Oleic acid (C18:1)	2.296 ± 0.003 a	2.3 ± 0.1 a
Linoleic acid (C18:2)	25.38 ± 0.04 a	24.2 ± 0.8 a
Linolenic acid (C18:3)	10.073 ± 0.009 a	9.4 ± 0.4 a
Total fatty acids	54.1 ± 0.1 a	51 ± 1 b

Values were reported in mean ± standard difference ($n = 8$). Different letters indicate significant differences between the samples ($p < 0.05$). (From Garde-Cerdán, Arias-Gil, et al., 2007)

(Quain, 1988). In a similar way, the processing treatment did not modify the long-chain saturated fatty acids (C14 and C16), which affect the degree of saturation of the plasma membrane of yeast (Ness, Adler, & Ness, 1984). Actually, must treatments should keep the total concentration of fatty acids unaltered since a low level could lead to slow fermentations and an increase of volatile acidity of wine because of an incorrect development of the yeast along the fermentation process.

Regarding the nitrogen content of the must, the PEF treatments applied did not affect the total concentration of amino acids (Table 5.3). On the other hand, the

Table 5.3 Concentration of amino acids (mg/L) measured on fresh and processed *Parellada* grape juice

Amino acid	Control	PEF
Proteic amino acids		
Arginine (Arg)	774 ± 69 a	772 ± 52 a
Proline (Pro)	119 ± 11 a	101 ± 6 a
Alanine (Ala)	82 ± 7 a	87 ± 6 a
Leucine (Leu)	28 ± 2 a	25 ± 2 a
Serine (Ser)	29 ± 3 a	29 ± 4 a
Threonine (Thr)	16 ± 1 a	14 ± 3 a
Tryptophan (Trp)	30 ± 2 a	46 ± 3 b
Glutamic acid (Glu)	31 ± 2 a	31 ± 2 a
Aspartic acid (Asp)	26 ± 2 a	23 ± 2 a
Valine (Val)	22 ± 2 a	30 ± 5 a
Asparagine (Asn)	24 ± 2 a	37 ± 3 b
Tyrosine (Tyr)	8.6 ± 0.3 a	11 ± 2 a
Histidine (His)	26.5 ± 0.9 a	33 ± 4 b
Methionine (Met)	28 ± 3 a	20 ± 4 a
Phenylalanine (Phe)	12.9 ± 0.7 a	18 ± 1 b
Isoleucine (Ile)	7.3 ± 0.2 a	6.3 ± 0.9 a
Lysine (Lys)	5.2 ± 0.5 a	8 ± 2 a
Glycine (Gly)	3.5 ± 0.3 a	3.2 ± 0.4 a
Non-proteic amino acids		
γ-Amino butyric acid (Gaba)	38 ± 3 a	32 ± 2 a
Cystathionine (Cyst)	37 ± 1 a	39 ± 12 a
Creatinine (Creat)	13 ± 1 a	12 ± 2 a
Ornithine (Orn)	9 ± 1 a	18 ± 3 b
Hydroxyproline (Hyp)	16 ± 2 a	20 ± 2 a
Phosphoserine (Pser)	8.3 ± 0.4 a	8.1 ± 0.5 a
Total amino acids	1394 ± 71 a	1424 ± 54 a

Values were reported in mean ± standard difference ($n = 8$)
Different letters indicate significant differences between the samples ($p < 0.05$). (From Garde-Cerdán, Arias-Gil, et al., 2007)

preservative treatment showed little effect on free amino acids content. In PEF-treated must, the concentrations of histidine, tryptophan, asparagine, phenylalanine, and ornithine were higher than in the control. These amino acids were probably released through the pores formed in the plasmatic membrane of the grape juice indigenous yeasts (Teissié et al., 2002). The conditions of PEF treatment could cause organelle disruptions, so vacuoles were destroyed too, allowing the proteases to have free access to the cytoplasmic enzymes (Harrison, Barbosa-Cánovas, & Swanson, 1997). These proteases could cause cellular protein degradation into smaller peptides and amino acids. It is noteworthy that PEF treatment did not reduce significantly the amino acid content because, as noted previously, it is directly linked with the fermentation rate, the yeast population, and with the global quality of the resulting wine (Arias-Gil, Garde-Cerdán, & Ancín-Azpilicueta, 2007; Garde-Cerdán et al., 2011; Garde-Cerdán & Ancín-Azpilicueta, 2008).

5.4.1.2 Concentration of Amino Acids, Biogenic Amines, and Volatile Compounds During Fermentation With and Without SO_2

The PEF-treated musts were inoculated with the yeast *S. cerevissiae* (NA33) and the vinification process was undertaken in the absence of and also with a reduced amount (20 mg/L) of SO_2. During the vinification process, the general enological parameters and the consumption of nitrogen compounds were evaluated (Garde-Cerdán, Marsellés-Fontanet, et al., 2007). The formation of biogenic amines (Garde-Cerdán et al., 2008) and volatile compounds (Garde-Cerdán, Marsellés-Fontanet, Arias-Gil, Ancín-Azpilicueta, & Martín-Belloso, 2008a) was also examined as these compounds determine the quality of the final wine.

It was observed that the highest assimilation of nitrogen-containing compounds was in the first half of alcoholic fermentation (Table 5.4), due to the growth phase of yeast (O'Connor-Cox & Ingledew, 1989). The ammonium nitrogen was practi-

Table 5.4 Nitrogenous fractions of the initial PEF-treated Parellada must, at 50 % of consumed sugars and of the final wines obtained from fermentation with SO_2 (20 mg/L) and without SO_2

Samples	Ammonium nitrogen (mg N/L)	Amino nitrogen (mg N/L)	Assimilable nitrogen (mg N/L)
Must			
	57.9 ± 0.5	300 ± 1	347 ± 1
50 % of the consumed sugars			
With SO_2	3.7 ± 0.7 a	129 ± 1 a	27 ± 1 a
Without SO_2	5 ± 0 b	175 ± 4 b	68 ± 8 b
Wine			
With SO_2	2.3 ± 0.6 a	71 ± 3 a	18.8 ± 0.6 a
Without SO_2	3.7 ± 0.7 a	100 ± 6 b	31.7 ± 0.7 b

All parameters are given with their standard deviation ($n = 8$)
Different letters indicate significant differences between the samples with and without SO_2 ($p < 0.05$). (From Garde-Cerdán, Marsellés-Fontanet, et al., 2007)

Table 5.5 Concentration of amino acids (mg/L) at 50 % of consumed sugars and in the final wines obtained from fermentation of PEF-treated Parellada must, with SO_2 (20 mg/L) and without SO_2

	50 % of consumed sugars		Wine	
Amino acids	With SO_2	Without SO_2	With SO_2	Without SO_2
Proteic amino acids				
Arginine (Arg)	3.0 ± 0.1 a	96 ± 7 b	3.03 ± 0.09 a	13 ± 1 b
Proline (Pro)	874 ± 33 a	919 ± 40 a	446 ± 12 a	591 ± 25 b
Alanine (Ala)	3.8 ± 0.1 a	7.2 ± 0.2 b	8.7 ± 0.2 a	11.9 ± 0.7 b
Leucine (Leu)	–	–	–a	1.2 ± 0.1 b
Serine (Ser)	3.1 ± 0.1 a	2.7 ± 0.2 a	2.4 ± 0.4 a	2.5 ± 0.6 a
Threonine (Thr)	–	–	–	–
Tryptophan (Trp)	–	–	–	–
Glutamic acid (Glu)	21 ± 1 a	34 ± 2 b	22.1 ± 0.2 a	22 ± 1 a
Aspartic acid (Asp)	3.2 ± 0.1 a	8.2 ± 0.5 b	4.2 ± 0.2 a	5.3 ± 0.3 a
Valine (Val)	–a	1.7 ± 0.1 b	–a	1.5 ± 0.2 b
Asparagine (Asn)	2.8 ± 0.1 a	7.4 ± 0.3 b	3.7 ± 0.3 a	7.2 ± 0.2 b
Tyrosine (Tyr)	–	–	–a	2.0 ± 0.1 b
Histidine (His)	1.7 ± 0.3 a	–b	1.9 ± 0.1 a	2.6 ± 0.2 b
Methionine (Met)	–a	5.3 ± 0.3 b	–a	4.0 ± 0.2 b
Phenylalanine (Phe)	0.81 ± 0.01 a	–b	–a	0.45 ± 0.03 b
Isoleucine (Ile)	–	–	–	–
Lysine (Lys)	8 ± 1 a	4.8 ± 0.4 b	3.0 ± 0.1 a	3.1 ± 0.2 a
Glycine (Gly)	4.8 ± 0.2 a	16 ± 2 b	3.0 ± 0.2 a	7.0 ± 0.7 b
Non-protein amino acids				
γ-Amino butyric acid (Gaba)	–	–	–	–
Cystathionine (Cyst)	15 ± 1 a	21 ± 2 b	11 ± 1 a	13 ± 1 a
Creatinine (Creat)	27 ± 1 a	36 ± 1 b	18.7 ± 0.1 a	27 ± 1 b
Ornithine (Orn)	1.9 ± 0.1 a	11 ± 1 b	1.0 ± 0.1 a	8 ± 1 b
Hydroxyproline (Hyp)	22 ± 2 a	18 ± 1 b	0.76 ± 0.03 a	2.6 ± 0.1 b
Phosphoserine (Pser)	6.7 ± 0.1 a	5.9 ± 0.1 b	2.9 ± 0.1 a	2.4 ± 0.2 b
Total amino acids	999 ± 33 a	1194 ± 41 b	532 ± 12 a	728 ± 25 b

The concentrations are shown with their standard deviations ($n = 8$). Different letters indicate significant differences between the samples with and without SO_2 ($p < 0.05$). (From Garde-Cerdán, Marsellés-Fontanet, et al., 2007)

cally entirely consumed in both fermentations. Amino nitrogen was reduced up to 76 % in the fermentation carried out with SO_2 and 67 % in absence of SO_2 (Table 5.4). Thus, the total assimilable nitrogen consumption was 328 mg N/L in the fermentation conducted with SO_2 and 315 mg N/L when SO_2 was not present.

Free amino acids are taken up by *S. cerevisiae* mainly during the first half of alcoholic fermentation (Tables 5.3 and 5.5). In addition, the use of amino acids, in this phase of the vinification, was higher in the fermentation conducted with SO_2 than in that performed without this additive. The most consumed amino acid during the first half of the alcoholic fermentation was arginine; this amino acid showed higher consumption in the fermentation conducted in presence of SO_2 than in the fermentation

without SO_2 (Table 5.5). The main nitrogen source for yeast during the fermentation was arginine and ammonium. Alanine was consumed to a large extent by yeasts during both types of fermentation. Leucine, threonine, tryptophan, tyrosine, isoleucine, γ-amino butyric acid, and citrulline were totally consumed in this phase of the alcoholic fermentation, as these amino acids are suitable nitrogen sources (Bell & Henschke, 2005). Glutamic acid was not consumed during the vinifications (Tables 5.3 and 5.5), even if this amino acid is a good source of nitrogen for the yeasts. Also, proline was not consumed in the initial stage of the alcoholic fermentation, as this amino acid is taken up by the yeasts only under poor nitrogen conditions (Arias-Gil et al., 2007; Garde-Cerdán, Marsellés-Fontanet, et al., 2007), and the ammonium ion in the medium can inhibit or repress the intake of proline (Ough & Stashak, 1974).

In the second half of alcoholic fermentation, the total amino acid consumption was the same in both types of fermentation, so that the level of SO_2 did not influence the amino acids intake (Table 5.5). The decrease of total amino acids was lower than that achieved in the first half of fermentation. In contrast to the first stage, the most consumed amino acid was proline (Table 5.5). Yeasts probably used this amino acid in this stage of alcoholic fermentation because there were less nitrogen sources than at the beginning of the fermentation. At the end of the vinifications, the wine obtained from the fermentation carried out with SO_2 had a lower concentration of amino acids than that obtained from the fermentation without any addition of SO_2.

Biogenic amines are aliphatic, aromatic, or heterocyclic organic bases of low molecular mass that occur in plants and in fermented foods. These compounds are undesirable in all foods and beverages because if consumed at an excessively high concentration, they may induce headaches, respiratory distress, heart palpitation, hyper-hypotension, and several allergenic disorders in humans (Silla-Santos, 1996). Moreover, polyamides, spermine, spermidine, and putrescine can react with nitrous acid and its salts to form nitrosamines, compounds of known carcinogenic action (Silla-Santos, 1996). Volatile amines can have an influence on wine aroma. Due to the acidic pH of the wine, these amines occur as odourless salts, but in the mouth they are partially neutralized and their flavour becomes apparent (Lehtonen, 1996). Fig. 5.2 shows the evolution on the concentration of biogenic amines during the alcoholic fermentations conducted with and without SO_2. In the initial musts, these nitrogen compounds were not found. The amines detected in the wines were putrescine, spermine, phenethylamine + spermidine, dimethylamine, and ethylamine; while histamine, tyramine, cadaverine, amylamine, hexylamine, diethylamine, isopropylamine, isobutylamine, and pyrrolidine were not synthesized during the alcoholic fermentation. The concentration of biogenic amines in the wines was very low, which indicates that the inoculated yeasts did not tend to produce these nitrogen compounds through alcoholic fermentation (Garde-Cerdán, Arias-Gil, et al., 2008). The most abundant biogenic amine was putrescine (Fig. 5.2a), since it is known that this compound is the major amine in the wines (Bover-Cid, Izquierdo-Pulido, Mariné-Font, & Vidal-Carou, 2006; Garde-Cerdán et al., 2014; Lehtonen, 1996). No differences were observed in the profile and concentration of these amines in function of the level of SO_2. This indicates that the SO_2 did not influence the synthesis of these nitrogen compounds.

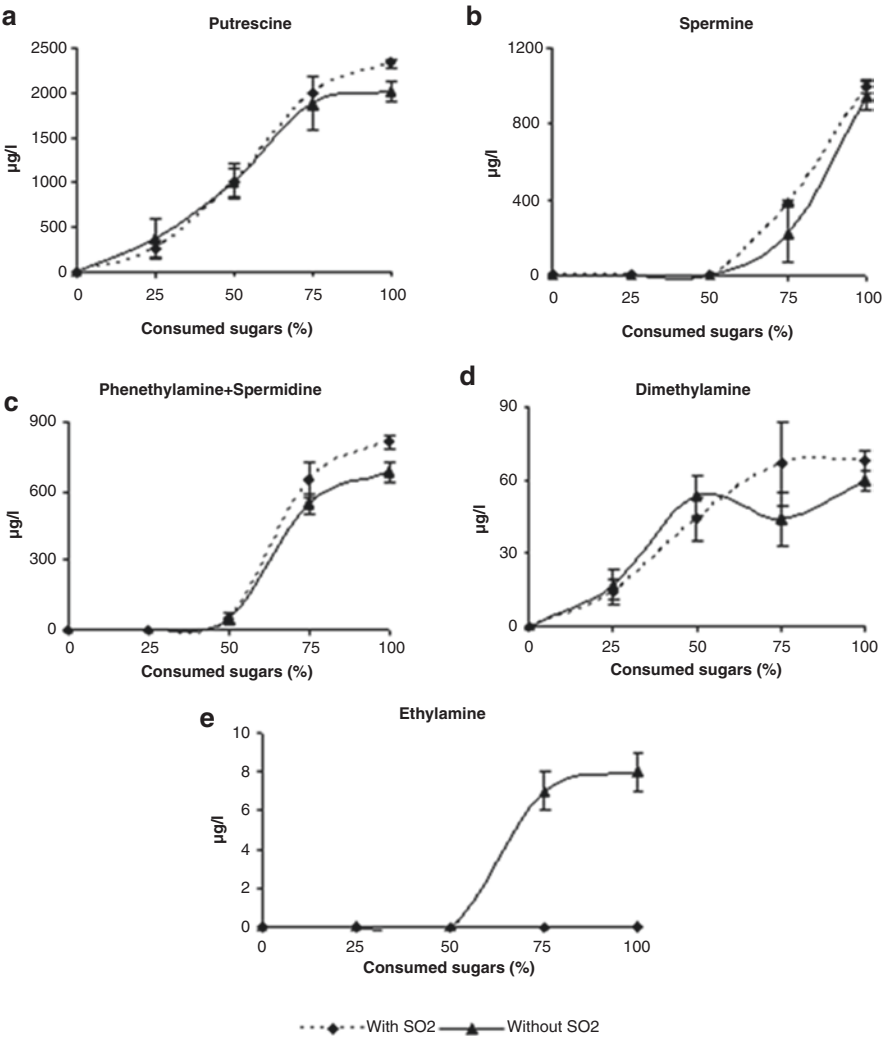

Fig. 5.2 Evolution of biogenic amines (µg/L) during the Parellada wine alcoholic fermentations with (20 mg/L) and without SO_2. All parameters are given with their standard deviation ($n=4$) (Garde-Cerdán, Arias-Gil, et al., 2008)

The fermentative volatile compounds are responsible for wine aroma, hence they determine the organoleptic characteristics of the final product. The synthesis of higher alcohols was higher during the first half of the fermentation (Fig. 5.3), since the consumption of amino acids was greater at this stage (Garde-Cerdán, Marsellés-Fontanet, et al., 2007). The vinification with SO_2 hardly modified their content after half-way fermentation, while the increase of higher alcohols in samples without this additive was smooth from the beginning to the end of the process. Final total alcohols content (160 mg/L) of wines fermented without SO_2 was around 20 % inferior

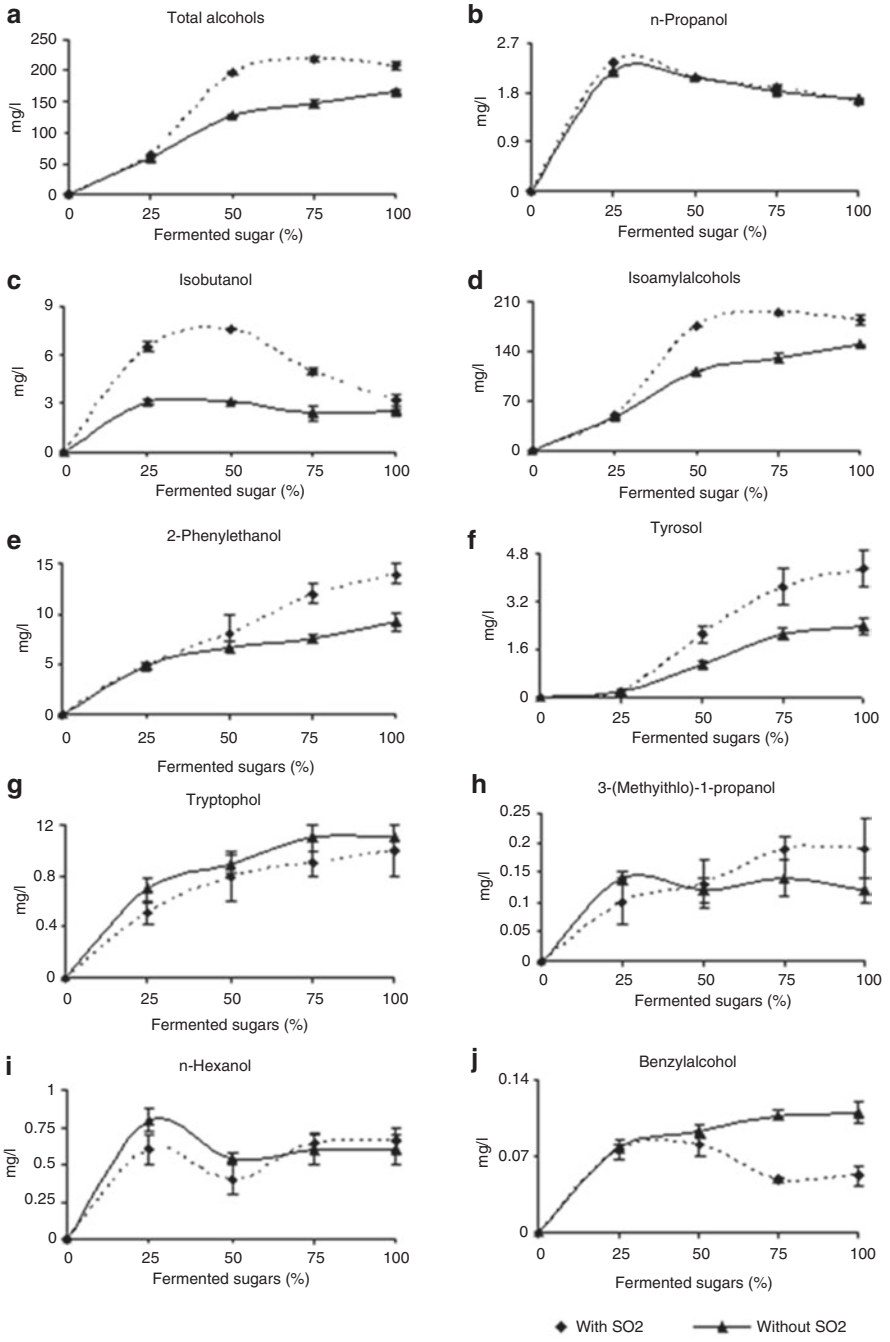

Fig. 5.3 Evolution of alcohols concentrations (mg/L) during the Parellada wine alcoholic fermentations with (20 mg/L) and without SO_2. All parameters are given with their standard deviation ($n = 8$) (Garde-Cerdán, Marsellés-Fontanet, et al., 2008a)

than in those fermented with this preservative. This value for total alcohols concentration was approximately half of the threshold for adversely affecting wine aroma that has been settled at above 300 mg/L (Rapp & Mandery, 1986). Yeasts consume amino acids to use them to form proteins as well as a nitrogen source. Although alcohols could be synthesized from sugars, the deamination of certain amino acids originates from the formation of higher alcohols. The amino acids precursors of isoamyl alcohols, 2-phenylethanol, and tyrosol (Fig. 5.3d–f), i.e., leucine, isoleucine, phenylalanine, and tyrosine, were completely consumed in both types of fermentations (Table 5.5). The greater synthesis of these four alcohols in the fermentation with SO_2 was probably because the yeast population in the fermentation with this additive presented more anabolic activity than that in the fermentation without SO_2 and used a greater quantity of these amino acids as a nitrogen source than in the fermentation without SO_2. The release of tryptophol, and 3-(methylthio)-1-propanol did not show significant differences between both fermentations (Fig. 5.3g, h), although the utilization of tryptophol precursor, i.e. tryptophan, was complete in both vinifications and the consumption of methionine, i.e. the precursor of 3-(methylthio)-1-propanol, was lesser in the fermentation without SO_2 than in that carried out with this additive (Table 5.5). The synthesis by the yeasts of *n*-propanol and *n*-hexanol was practically the same whether using SO_2 or not (Fig. 5.3b, i). The formation of these alcohols is not related with the anabolic routes of amino acids; the same occurs with benzyl alcohol. This latter compound was the only alcohol whose content was higher in the fermentation without SO_2 than in that with this additive (Fig. 5.3j).

Regarding esters produced during the alcoholic fermentation, the evolution of their concentration is shown in Fig. 5.4. Independently of the use of SO_2, the profile throughout the winemaking process for all of them was very similar, having a substantial increase after 25 % of consumed sugars followed by a gradual increase until reaching their maximum concentration at the end of the fermentation. This fact agrees with Jackson (2008) who reported that the formation of esters by the yeasts is inhibited by oxygen, which could be dissolved to some degree at the beginning of alcoholic fermentation. Moreover, in general, throughout the fermentation carried out without SO_2, the samples had a higher total esters level than those from the fermentation performed in the presence of this additive (Fig. 5.4a). However, only the concentration of ethyl hexanoate (Fig. 5.4e) showed differences between both types of fermentation. This agrees with the results found by Herraiz (1990). This author observed that ethyl hexanoate was synthesized in higher quantity in the fermentation made without SO_2 than in the fermentation carried out with SO_2.

Ethyl acetate is an ester that confers complexity to the wine aroma below 50 mg/L, while above 150 mg/L, it adds vinegary off-odours to the wine (Amerine & Roessler, 1983). Both types of fermentation yielded a similar final concentration of this ester, which was less than 50 mg/L (Lambrechts & Pretorious, 2000). The concentrations of isoamyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate (Fig. 5.4c, e–g), esters that play a major role in wine aroma, were above their threshold levels (1, 0.08, 0.58, and 0.51 mg/L, respectively) (Etiévant, 1991;

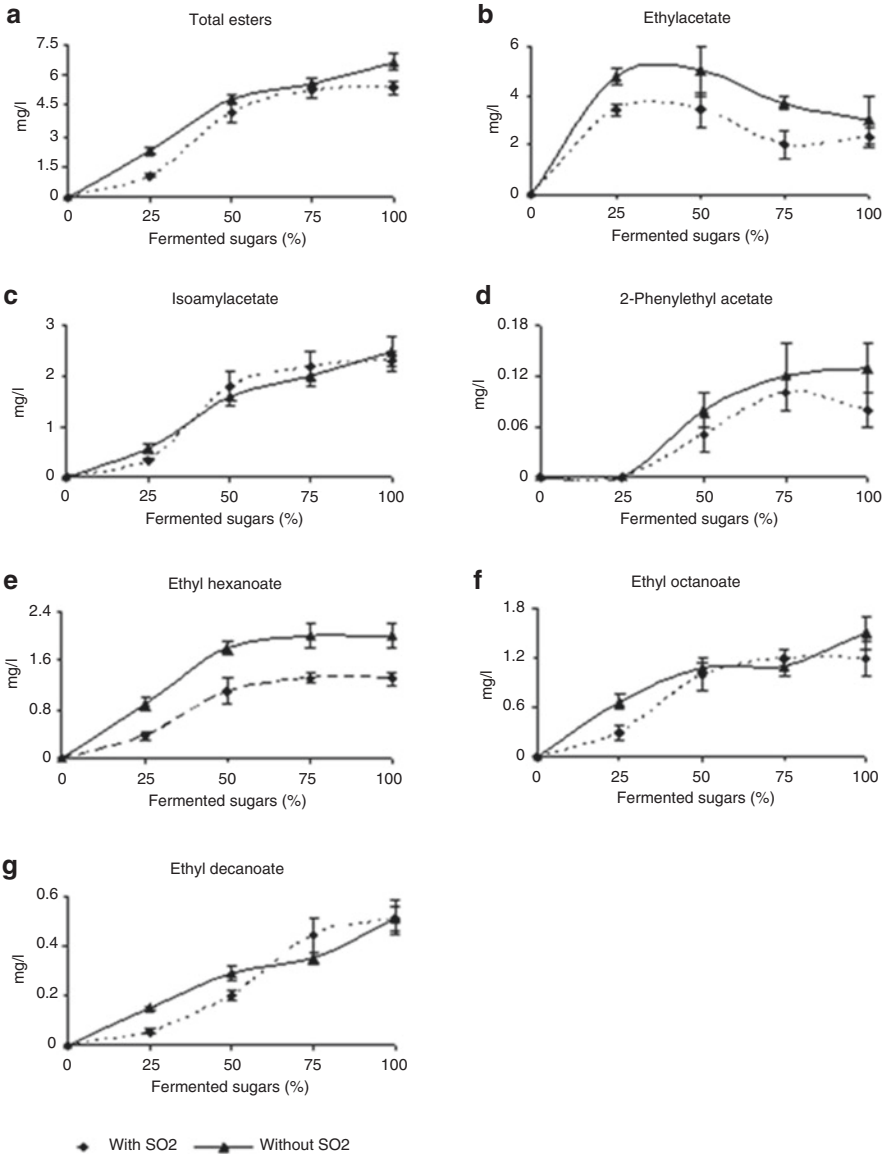

Fig. 5.4 Evolution of esters concentrations (mg/L) during the Parellada wine alcoholic fermentations with (20 mg/L) and without SO_2. All parameters are given with their standard deviation ($n = 8$) (Garde-Cerdán, Marsellés-Fontanet, et al., 2008a)

Simpson, 1979). Thus, the absence of SO_2 during the fermentation development should not decrease the wine quality.

Unlike the volatile compounds, concentrations of fatty acids throughout fermentations carried out with and without SO_2 were approximately the same (Fig. 5.5). The final concentration of fatty acids in the wines was 15 mg/L regardless of the

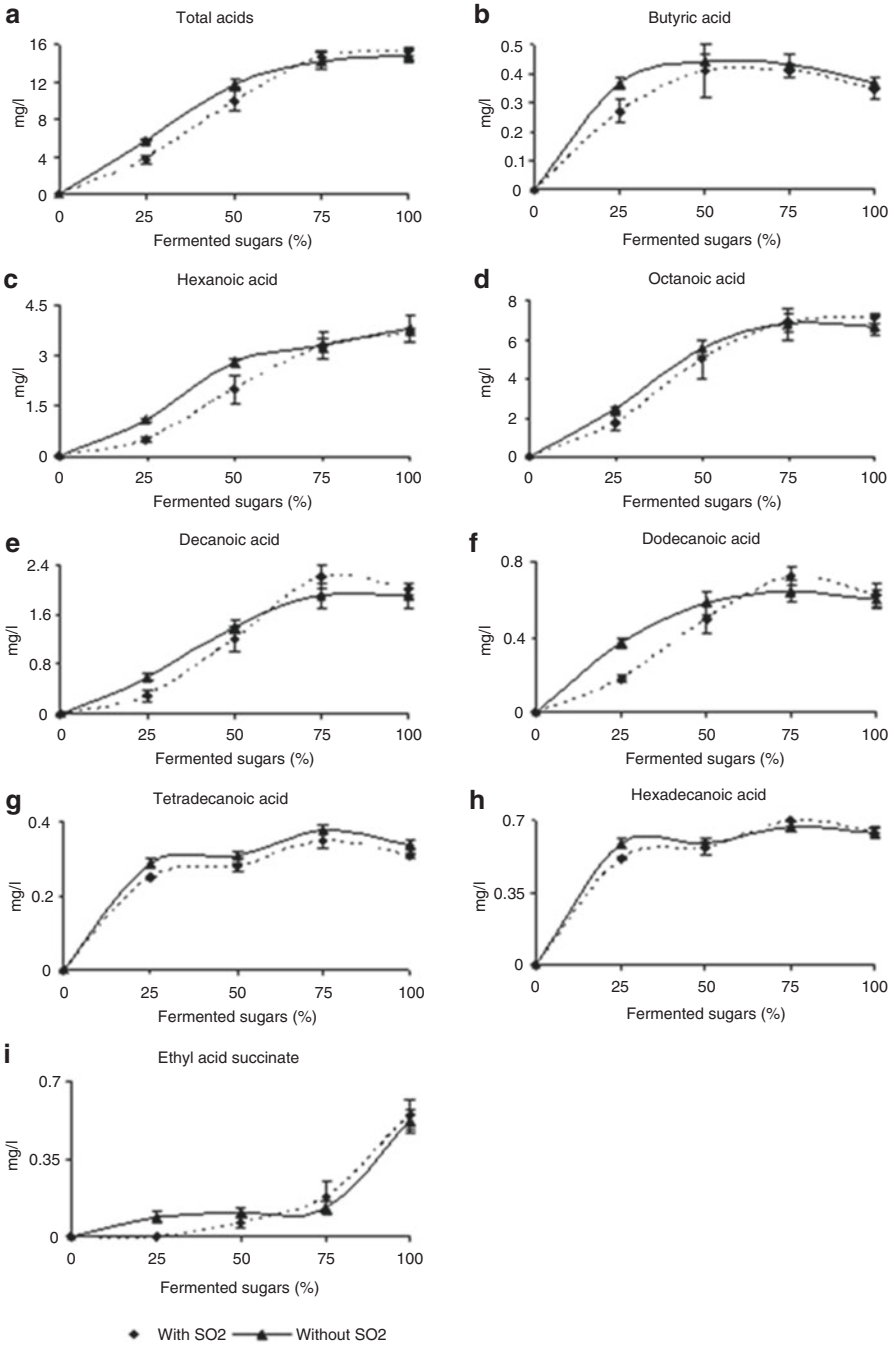

Fig. 5.5 Evolution of acids concentrations (mg/L) during the Parellada wine alcoholic fermentations with (20 mg/L) and without SO_2. All parameters are given with their standard deviation ($n = 8$) (Garde-Cerdán, Marsellés-Fontanet, et al., 2008a)

presence of SO_2. These compounds produce changes in wine flavour depending on their concentrations. It has been stated that fatty acids add a fresh flavour to wine, increasing the appreciation of other taste sensations, at a concentration around 20 mg/L, whereas higher values confer an unpleasant flavour to the wines (Pozo-Bayón et al., 2005). Therefore, the results obtained indicate that the elimination of SO_2 during the alcoholic fermentation did not produce any detrimental effect to the typical notes that fatty acids confer to wine flavour when must is stabilized by PEF, as the total concentration of fatty acids in final wines was below 20 mg/L.

5.4.1.3 Volatile Compounds During Ageing in Bottles Without SO_2

At the end of the vinification process, the wines obtained from the fermentation of the must treated by PEF and elaborated in absence of SO_2 were aged in bottle in a dark place at room temperature (RT) or at a controlled temperature of 5 °C, for 6 months. Non-treated must of the same variety was fermented with the addition of SO_2 (50 mg/L) following the standard procedure for white wine production; the fermentation of these musts begun and developed spontaneously by the native yeast species. These wines were aged simultaneously with the PEF-treated wines under the same conditions. The evolution of the concentration of volatile compounds was examined during the ageing process of both wines at the end of the fermentation and at 3 and 6 moths of ageing (Garde-Cerdán, Marsellés-Fontanet, Arias-Gil, Ancín-Azpilicueta, & Martín-Belloso, 2008b).

During 6 months of storage, the concentration of total alcohols increased slightly in the wines aged in bottle at RT, while it did not change in the wines that remained at 5 °C (Fig. 5.6a). As the wine had residual concentration of amino acids, they could be transformed into the corresponding alcohols at RT. The concentration of total alcohols in the wines was below 300 mg/L, so these compounds did not contribute to wine aroma. The concentration of isobutanol increased in the wine aged at RT, while its concentration diminished in the wine that remained at 5 °C (Fig. 5.6b). *n*-Propanol and isoamyl alcohols concentrations scarcely changed during the time of the wines in the bottles (Fig. 5.6c, d). The levels of these alcohols were slightly higher in the wine that remained at RT than in that stored at low temperature. Isoamyl alcohols were found in higher concentration in wine obtained from inoculated fermentation (Fig. 5.6d) than in wine obtained from standard fermentation (Table 5.6). This was probably because *Saccharomyces* yeasts are good producers of these alcohols (Romano, Fiore, Paraggio, Caruso, & Capece, 2003). The concentration of 2-phenylethanol increased in a similar way in both wines during the ageing in bottles, regardless of temperature (Fig. 5.6e). This compound is important for wine quality since it gives rose aroma (Etiévant, 1991), and since it was above its threshold level (14 mg/L; Ferreira, López, & Cacho, 2000), it could contribute in a positive way to wine aroma. The concentration of 2-phenylethanol was higher in the wine obtained through standard fermentation (Table 5.6) than in that obtained from inoculated fermentation (Fig. 5.6e), given that the non-*Saccharomyces* indigenous yeasts are good producers of this higher alcohol (Garde-Cerdán & Ancín-Azpilicueta, 2006). The concentration of tyrosol and tryptophol diminished in both

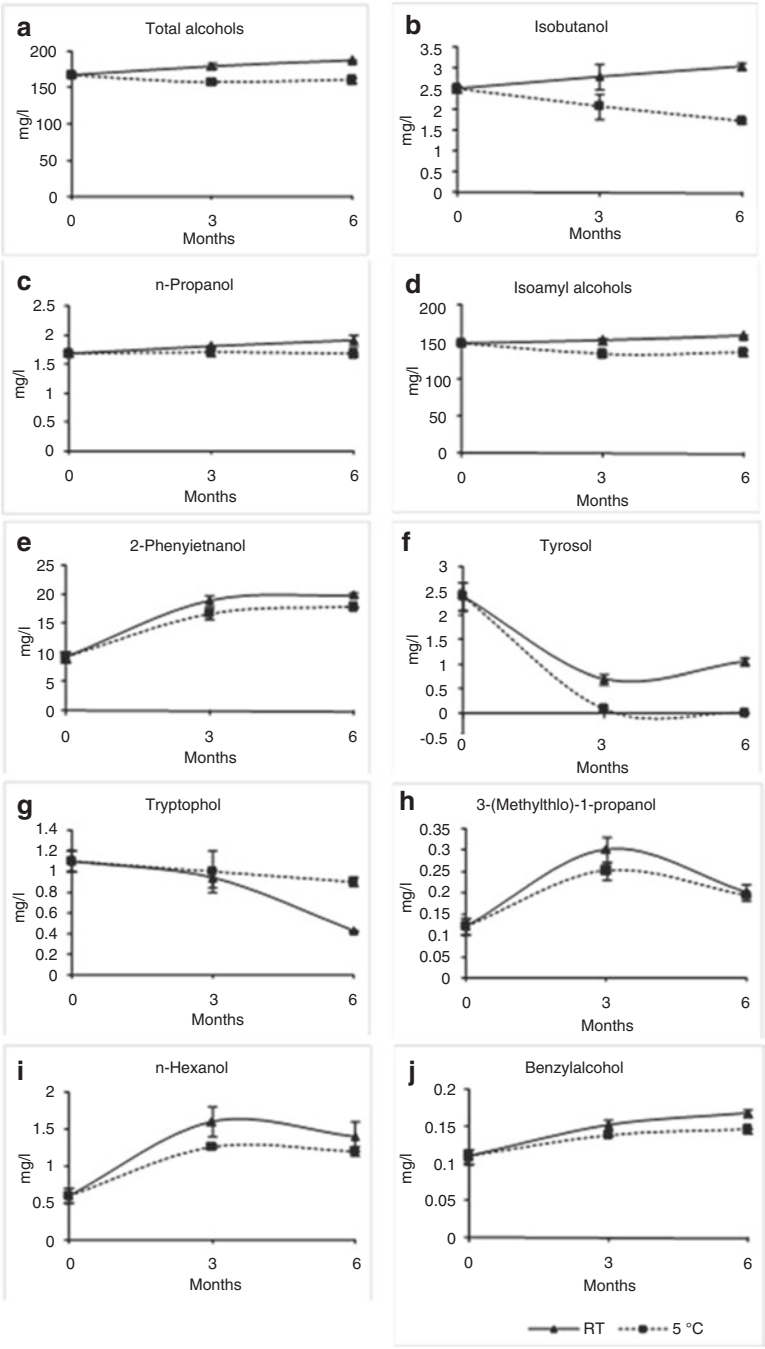

Fig. 5.6 Evolution of alcohols concentrations (mg/L) in the wines obtained from Parellada must stabilized by PEF and aged in bottles at room temperature (weighted average temperature, 23 °C) and at low and controlled temperature (5 °C). All parameters are given with their standard deviation ($n = 8$) (Garde-Cerdán, Marsellés-Fontanet, et al., 2008b)

wines, although in a different way; at the end of the ageing, the concentration of tyrosol was higher in the wines that remained at RT while the concentration of tryptophol was higher in the wines maintained at low temperature (5 °C) (Fig. 5.6f, g). However, the concentration of these two alcohols increased during the ageing time in the wines from standard fermentation (Table 5.6). The concentration of 3-(methylthio)-1-propanol, *n*-hexanol, and benzyl alcohol evolved in a similar way in both wines (Fig. 5.6h–j).

The level of total esters hardly suffered modifications in the wine that remained in bottles at low temperature (5 °C), while it increased in a significant way in the wine aged at RT (Fig. 5.7a). Something similar happened with the wine obtained from standard winemaking process (Table 5.6). The concentration of ethyl acetate diminished in both wines during their permanency in bottles at different temperatures (Fig. 5.7b). At the end of the ageing, the concentration of this ester was similar in both wines and very inferior to the concentration that is negative to wine aroma. Ethyl acetate was synthesized at high quantity in the standard fermentation (Table 5.6), probably due to the fact that non-*Saccharomyces* yeasts are greater producers of this compound than *Saccharomyces* yeasts (Plata, Millán, Mauricio, & Ortega, 2003). 2-Phenylethyl acetate was only formed in the standard fermentation (Table 5.6). Rojas, Gil, Piñaga, and Manzanares (2003) found that non-*Saccharomyces* yeasts are good producers of this ester during alcoholic fermentation. The concentration of the esters that exert a high influence on wine aroma, i.e. isoamyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate, diminished with the storage time in the samples that remained at RT more than in those that stayed at low temperature (5 °C) (Fig. 5.7c–f), with the exception of ethyl octanoate whose evolution was similar in both wines, regardless of temperature. Isoamyl acetate, ethyl hexanoate, and ethyl octanoate were synthesized in higher quantities in the fermentation of stabilized and inoculated must than in the standard fermentation (Table 5.6). However, the concentration of ethyl esters of organic acids, i.e. ethyl acetate, diethyl succinate, monoethyl succinate, and diethyl malate, increased in an important way in the wines aged in bottle at RT, while in the wines that remained at 5 °C the concentration of these compounds increased slightly or did not change (Fig. 5.7g–j). These esters, except monoethyl succinate, were not found in the wine obtained from standard fermentation (Table 5.6). The ethyl esters of fatty acids and the acetate ester of higher alcohols were hydrolyzed with the ageing time, while the ethyl esters of organic acids were formed during the storage time in the bottles. These results agree with those found by other authors (Ferreira, Escudero, Fernández, & Cacho, 1997; Ramey & Ough, 1980). Both processes, esterification of fatty acids and hydrolysis of acetate esters, were favoured by an increase in the temperature (Fig. 5.7).

Total fatty acids concentration increased during the ageing of the wines in bottles, where there was a higher concentration in the wines that remained at 5 °C than in the ones aged at RT (Fig. 5.8a). The contents of total acids and hexanoic, octanoic, and decanoic acids in the wine obtained from standard fermentation (Table 5.6) were lower than those found in the wine made from stabilized and inoculated must (Fig. 5.8). This could be due to the fact that the *Saccharomyces* inoculated yeasts are

Table 5.6 Evolution of volatile compounds concentrations (mg/L) of Parellada wines obtained from standard winemaking process and aged in bottles at room temperature (weighted average temperature, 23 °C) and at low and controlled temperature (5 °C)

		3 months		6 months	
	Wine	RT	5 °C	RT	5 °C
Esters					
Total esters	10 ± 1	16 ± 1	17.1 ± 0.4	20 ± 1	15 ± 2
Ethyl acetate	7 ± 3	7.5 ± 0.4	8 ± 1	8 ± 2	6 ± 1
2-Phenylethyl acetate	9 ± 1	7.3 ± 0.2	12.8 ± 0.2	2.0 ± 0.3	10 ± 2
Isoamyl acetate	0.6 ± 0.1	0.62 ± 0.02	0.9 ± 0.2	0.43 ± 0.02	0.9 ± 0.2
Ethyl octanoate	0.017 ± 0.003	0.04 ± 0.01	0.026 ± 0.003	0.025 ± 0.008	0.06 ± 0.01
Ethyl decanoate	0.20 ± 0.03	0.144 ± 0.009	0.22 ± 0.05	0.12 ± 0.01	0.17 ± 0.01
Monoethyl succinate	0.45 ± 0.06	8 ± 1	3.2 ± 0.3	17 ± 1	4.31 ± 0.04
Alcohols					
Total alcohols	140 ± 2	177 ± 2	184 ± 1	181 ± 5	191 ± 5
Isobutanol	3.2 ± 0.2	1.9 ± 0.3	2.4 ± 0.2	2.00 ± 0.01	3.0 ± 0.1
n-Propanol	2.0 ± 0.1	1.34 ± 0.01	1.484 ± 0.003	1.63 ± 0.03	1.79 ± 0.01
Isoamyl alcohols	117 ± 2	129.7 ± 0.5	135 ± 1	124 ± 1	135 ± 3
2-Phenylethanol	13.7 ± 0.8	28 ± 2	30.1 ± 0.3	29 ± 1	31 ± 2
Tyrosol	1.1 ± 0.2	3.8 ± 0.6	2.90 ± 0.02	5.3 ± 0.6	2.5 ± 0.3
Tryptophol	1.9 ± 0.3	10.47 ± 0.01	8.9 ± 0.1	17 ± 5	15 ± 4
3-(Methylthio)-1-propanol	0.15 ± 0.02	0.41 ± 0.01	0.7 ± 0.1	0.6 ± 0.2	0.7 ± 0.1
n-Hexanol	0.9 ± 0.2	1.9 ± 0.1	2.6 ± 0.1	1.9 ± 0.2	2.1 ± 0.1
Benzyl alcohol	0.074 ± 0.008	0.171 ± 0.006	0.19 ± 0.01	0.195 ± 0.005	0.19 ± 0.03
Acids					
Total acids	1.1 ± 0.1	2.0 ± 0.1	2.24 ± 0.04	1.8 ± 0.1	2.46 ± 0.04
Hexanoic acid	0.27 ± 0.05	0.52 ± 0.04	0.671 ± 0.003	0.71 ± 0.03	0.75 ± 0.02
Octanoic acid	0.45 ± 0.04	0.61 ± 0.04	0.626 ± 0.001	0.59 ± 0.05	0.89 ± 0.02
Decanoic acid	0.4 ± 0.1	0.9 ± 0.1	0.94 ± 0.04	0.53 ± 0.07	0.82 ± 0.03

All parameters are given with their standard deviation (n = 8). (From Garde-Cerdán, Marsellés-Fontanet, et al., 2008b)

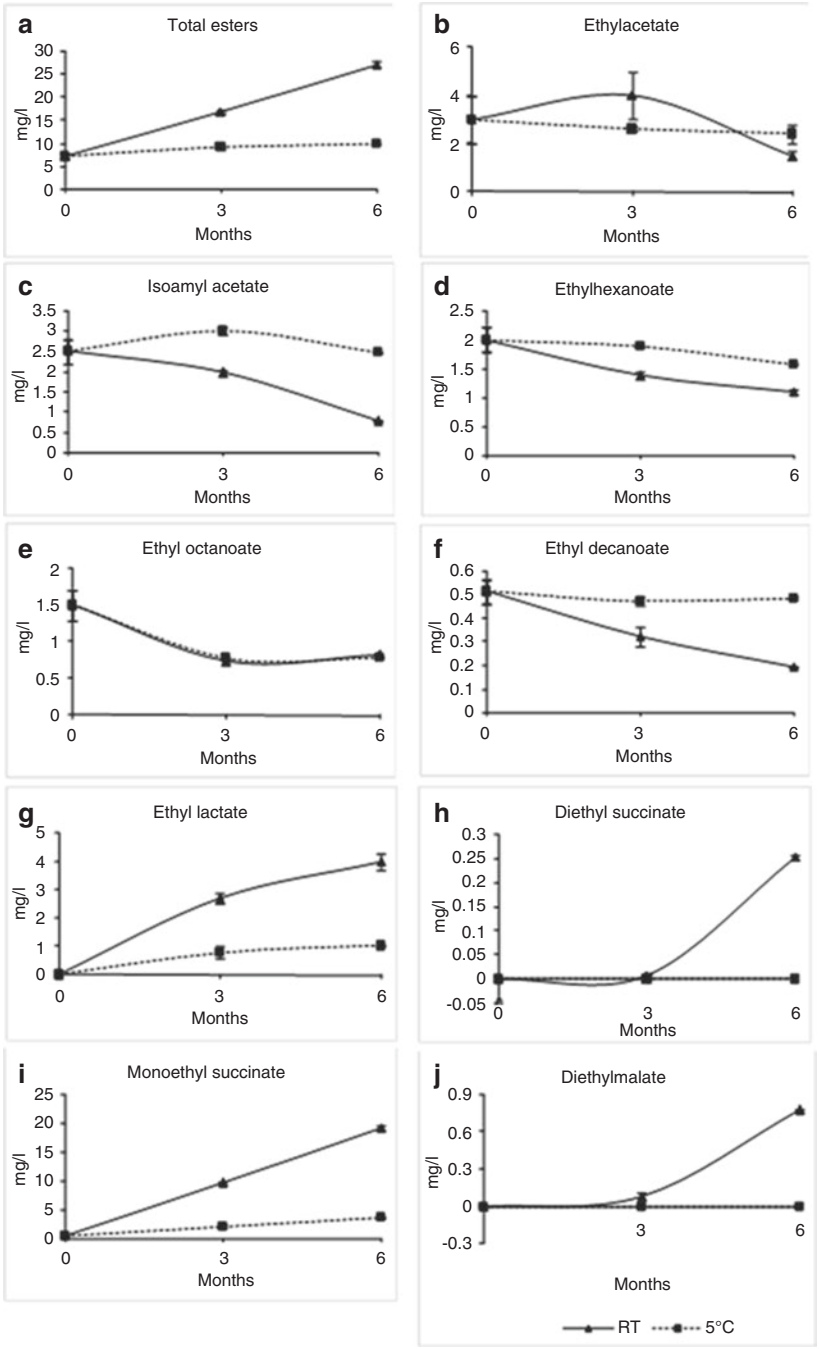

Fig. 5.7 Evolution of esters concentrations (mg/L) in the wines obtained from Parellada must stabilized by PEF and aged in bottles at room temperature (weighted average temperature, 23 °C) and at low and controlled temperature (5 °C). All parameters are given with their standard deviation ($n = 8$) (Garde-Cerdán, Marsellés-Fontanet, et al., 2008b)

good producers of these acids (Garde-Cerdán, Marsellés-Fontanet, et al., 2008a). Butyric and hexanoic acids increased their concentration in a similar way in both wines with the ageing time (Fig. 5.8b, c). The contents of octanoic and decanoic acids increased in both wines up to the 3 months of permanency in bottle and, later on, their concentrations diminished in such a way that the wines aged at low temperature presented higher concentration of these acids than those that remained at RT (Fig. 5.8d, e). The concentration of dodecanoic acid changed little in the wine aged at RT and increased in the wine that remained at 5 °C (Fig. 5.8f). The concentration of tetradecanoic acid diminished slightly in the wine aged at RT and there was almost no change in the wine that remained at low temperature (Fig. 5.8g). The concentration of hexadecanoic acid increased in both wines, although to a higher extent at the end of the ageing in the wine that stayed at 5 °C (Fig. 5.8h). These last three acids were not found in the wine obtained from standard fermentation (Table 5.6).

Consequently, from the point of view of aromatic quality, the conservation of white wines obtained by fermentation of must processed with PEF and aged in bottles without the addition of SO_2 was better at 5 °C than at RT.

5.4.1.4 Reduction of Yeast Population in Winemaking

To the authors' knowledge, there is only one other study in the literature where pulsed electric field treatments have been employed in substitution of SO_2 for wine production. Thus, Delsart et al. (2015) carried out a study related to the production of sweet white wines using PEF or SO_2 at different concentrations (100, 250 or 500 mg/L) to treat the wines after 5 days of fermentation. In this study, the inactivation of fermentative yeast by PEF and by adding SO_2 at different concentrations was examined as well as the browning of the respective sweet wines. The results showed that the highest inactivation for total yeasts was 3 log units and 8 log units with PEF or 250 mg/L of SO_2, respectively. The population of non-*Sacharomyces* yeast was reduced in 4 log units using PEF treatment and 7 log units using SO_2. Thus, SO_2 was more effective than PEF for the inactivation of fermentative yeast. However, the wine browning was less pronounced for the samples treated by PEF in comparison to SO_2 treatments. Therefore, the authors conclude that PEF seems to be the most suitable alternative technique to SO_2 addition in sweet wine production. The evaluation of further studies in relation with the phenolic and volatile composition and the sensory analysis of PEF-treated sweet wines were recommended by the authors in order to confirm the benefits of PEF over the addition of SO_2.

In view of the results, PEF treatment inactivated the microbial population of the treated samples without causing detrimental effects to the musts, neither did the fermentation kinetics on the resulting wines, so, in this respect, the technique is a feasible candidate to substitute SO_2 in winemaking. However, the high electric field strength necessary for the efficient inactivation of microorganisms and its consequent elevated cost has prevented the use of PEF at industrial scale for sterilizing purposes (Puértolas et al., 2010a).

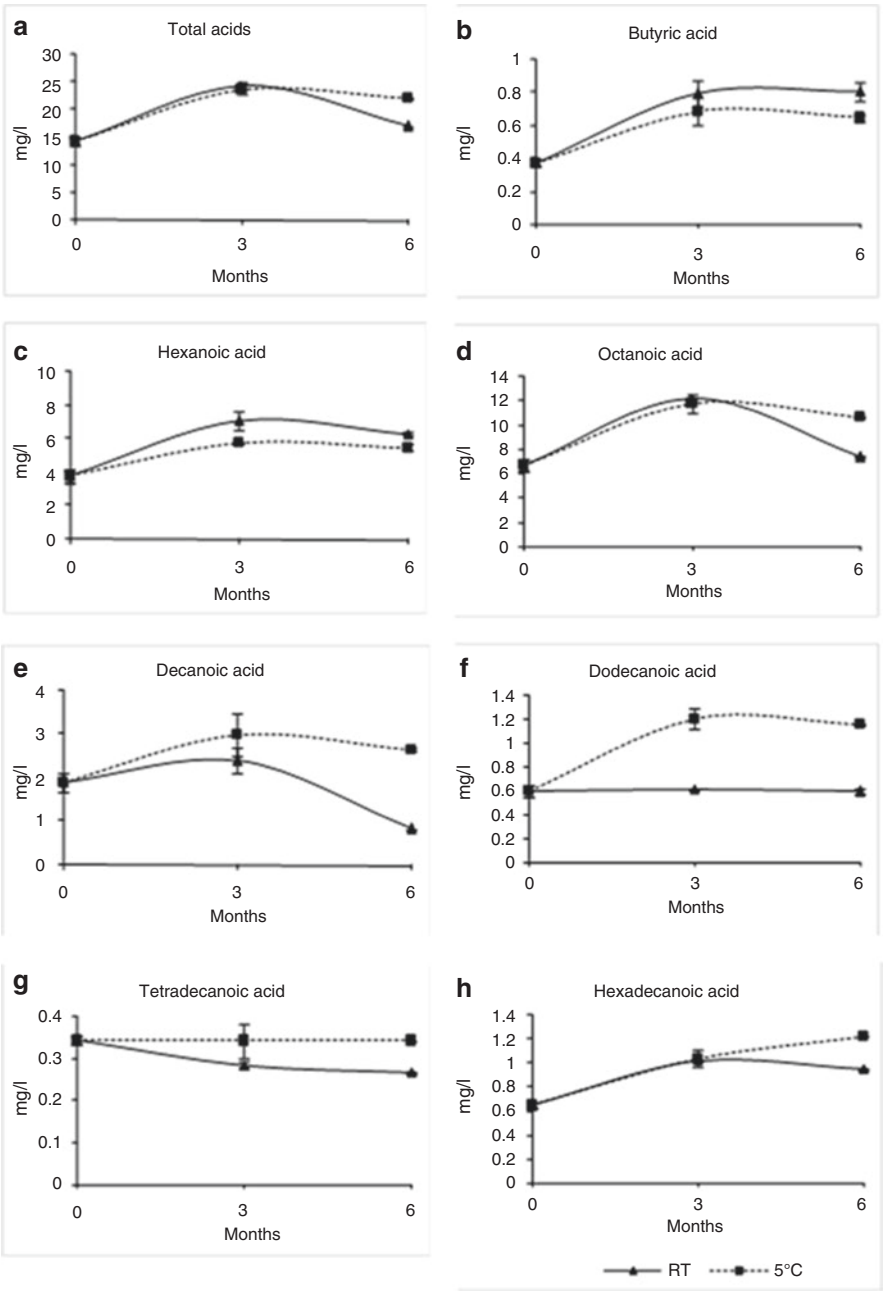

Fig. 5.8 Evolution of acids concentrations (mg/L) in the wines obtained from Parellada must stabilized by PEF and aged in bottles at room temperature (weighted average temperature, 23 °C) and at low and controlled temperature (5 °C). All parameters are given with their standard deviation ($n = 8$) (Garde-Cerdán, Marsellés-Fontanet, et al., 2008b)

5.4.2 Extraction of Compounds from Grape Skins

Phenolic extraction from grape skins in wine production is a diffusion process, which strongly depends on the grape skin cell permeability and on the winemaking technology employed (Luengo et al., 2014; Sacchi, Bisson, & Adams, 2005). The extraction of phenolic compounds is generally obtained by leaving grape skins in contact with the must during the fermentative process. However, to obtain deeply coloured and full bodied wines, maximum phenol extraction is required. For certain grape varieties, the increase of phenolic extraction is achieved by extending the maceration period beyond the fermentative process (Luengo et al., 2014). This practice, in turn, has two major drawbacks. On the one hand, the wineries have to increase the number of fermentation tanks, which increases the cost inputs on wineries. On the other hand, proanthocyanidins over-extraction from grape seeds, strength which is favoured at high ethanol concentrations, may result in wines with excessive bitterness and astringency (López, Puértolas, Condón, Álvarez, & Raso, 2008a).

The effect of PEF treatments on the increase of the permeability of eukaryotic cells has been shown to aid in a significant manner the mass transfer between vegetable tissues and juices (Asavasanti, Ersus, Ristenpart, Stroeve, & Barrett, 2010; Brianceau, Turk, Vitrac, & Vorobiev, 2014; Donsì, Ferrari & Pataro, 2010). These treatments have been applied to red and rosé wine production with extractive purposes. PEF applied on grape skins has been shown to increase the extraction rate and amount of phenolic compounds (López-Alfaro et al., 2013; López-Giral et al., 2015; Puértolas et al., 2010a, 2010b), the concentration of primary aromas (Garde-Cerdán et al., 2013), and also the must yield (Donsì, Ferrari & Pataro, 2010; Praporscic, Lebovka, Vorobiev, & Mietton-Peuchot, 2007). In comparison with yeast and bacteria, the electroporation of large-size eukaryotic cells is obtained at milder electric strengths and low energy inputs (Puértolas et al., 2010a). Thus, the subsequent cost of PEF for extractive purposes is lower than that of pasteurization. Furthermore, the mild electric field strengths employed are not enough to infer the permeabilization of cell walls and membranes of grape seeds, which reduced the risk of proanthocyanidins over extraction (López et al. 2008a; Luengo et al., 2014).

The reduction of fermentation-maceration period to obtain red wines derived from Garnacha grapes was demonstrated by Luengo et al. (2014). The PEF treatment was applied in continuous flow, in a manner that the mixture of crushed grapes and must were pumped through a collinear treatment chamber, equipped with tubular electrodes, to the maceration tank. The treatment consists of the application of 20 pulses of square wave shape and 3 μs of pulse width. The electric field applied was of 4 kV/cm at 250 Hz of frequency. The chromatic characteristics of the treated must, such as the colour intensity (CI), the total anthocyanin content (TAC), and the total polyphenol index (TPI), were higher than those of non-treated samples at the beginning of the maceration-fermentation process. After 5 days of fermentation, CI and TAC reached maximum values, which were higher in PEF-treated must than in

controls. TPI reached maximum values at day 7 of maceration-fermentation process and these values were also higher in the treated samples than in control samples. At that day, grape skins were removed for some of the maceration tanks. At the end of the process (14 days), the values of CI, TAC, and TPI of wines from treated grape skins did not present significant differences with respect to controls. Thus, it was demonstrated that, by applying PEF on Garnacha grapes, the electroporation of vegetable skin cells permitted the same level of phenolic compounds extraction than in control grapes, but in half of the time. The rest of the physicochemical characteristics of the treated and untreated samples were similar after the treatment and at the end of the fermentation process (Luengo et al., 2014). Furthermore, when the wines were evaluated by a sensory panel, wines derived from PEF-treated grape skins and 7 maceration days were preferred over control wines and wines derived from PEF-treatment macerated for 14 days. This suggest that, for this variety, extended maceration times infer a detriment in the wine bouquet, consequently PEF treatment can be a potential alternative process to reduce maceration times and in turn improve the organoleptic characteristics of Garnacha wines.

It is noticeable that the grape variety and the treatment parameters determine the PEF effects for colour extraction. Thus, in a study performed by Puértolas et al. (2010b), 50 square wave pulses of 3 μs width at 122 Hz of frequency and electric fields of 0.5, 2, and 7.5 kV/cm were applied, in a continuous flow mode, to respective mixtures of crushed grapes of Cabernet sauvignon, Syrah, and Merlot varieties before the maceration-fermentation process. The extraction rates, concentration of anthocyanins, and total phenols were higher in PEF-treated samples than in controls. Those compounds were more effectively extracted at the higher electric field intensity of 7.5 kV/cm. The maximum values were reached after 50 h of fermentation in treated samples and after 96 h in control samples. Different extraction efficiencies were observed in the varieties studied. Thus, applying PEF, phenolic compounds were extracted more efficiently from Cabernet sauvignon variety than from Syrah and Merlot varieties (Puértolas et al., 2010b). The authors suggested that different cell morphologies and cell envelope composition may lead to significant differences on the extractive efficiency of PEF treatments for the varieties studied. Differences on the extraction efficiencies of phenolic compounds, inferred by PEF treatment applied at different electric strength, were also observed by Donsì, Ferrari, Fruilo, and Pataro (2010) for Piedirosso and Aglianico grape varieties. In this study, the permeabilization of cell skins was measured after different PEF treatments. It was observed that Piedirosso grapes were more sensitive to PEF treatment than Aglianico grapes, since permeabilization of the later was achieved at higher electric field strengths and relative energies. Furthermore, it was observed that efficiency of cell permeabilization was more favoured by applying high energy inputs than by the increments of the electric field strength.

Different responses to PEF treatments by diverse grape varieties were also found by López et al. (2008a). In this study after the application of PEF treatments to Garnacha, Mazuelo, and Graciano grape skins, it was observed that Mazuelo variety was more sensitive to the Electric field applied than the other varieties. Thus, the higher the electric field strength, the higher the values of CI, TAC and

TPI in Mazuelo wines. The treatment was applied to the grape skins in batch mode with 50 pulses of exponential decay at a frequency of 1 Hz and different electric field strengths of 2, 5, and 10 kV/cm, with specific energies of 0.4, 1.8, and 6.7 kJ/kg, respectively. After the treatment, the grape skins were mixed with the respective must for the maceration-fermentation process. Although, in general, wines from treated samples presented higher values of CI, TPI, and TAC than respective controls, PEF treatments were more effective on the extraction of phenolic compounds for Mazuelo grapes since control wines from this variety presented the lower CI, TPI and TAC values in comparison with other controls. Thus, the authors suggested the benefits of PEF on the vinification when the treatment is applied to grape varieties of poor colour (López et al. 2008a). The same research group investigated the application of PEF at different electric field strengths to extract phenolic compounds from Tempranillo grape variety (López, Puértolas, Condón, Álvarez, & Raso, 2008b). Pulses were applied in batch mode at electric field strengths of 5 and 10 kV/cm with respective specific energies of 1.8 kJ/kg and 6.7 kJ/kg. For this variety, field intensities of 10 kV/cm enhanced the extraction of total anthocyanins and increased the CI of final wines. However, maximum TPI was achieved at electric field strengths of 5 kV/cm, and high electric field strengths did not increase this parameter (López, et al. 2008b).

Delsart et al. (2014) and Cholet et al. (2014) evaluated the influence of treatment parameters on the extractive effect of PEF by examining the structural and chemical changes of grape skin cells. In these studies, it was observed that structural changes of grape cells caused by PEF were a function of the electric field applied and the treatment time. At the same time, the mass transfer process depended on the structural variations of the cells (Delsart et al., 2014). Thus, two different PEF treatments of square wave shape (4 kV/cm, 1 ms treatment time, 4 Wh/kg, and 0.7 kV/cm, 200 ms treatment time, 31 Wh/kg) were applied to crushed Cabernet sauvignon grapes before the maceration-fermentation process. In this study, it was observed that the treatment at low electric field strength and long duration (0.7 kV/cm, 200 ms) promoted the disaggregation of parietal tannins. It also induced important structural changes on cell walls and the electroporation of cell membranes. This permitted the release of disaggregated tannins and other phenols into the media (Delsart et al., 2014). Therefore, the obtained wines presented the highest concentration of anthocyanins and proanthocyanidins and also the highest CI and TPI in comparison with the control wines and wines elaborated from grapes treated at higher electric field treatment. The treatment at high electric field strength and short duration (4 kV/cm, 1 ms) promoted the disaggregation of vacuolar tannins and cell layer distension. However, cell wall damage was minimal and consequently the disaggregated tannins were accumulated on cell walls but their release to the media was limited (Cholet et al., 2014; Delsart et al., 2014). Thus, although the wines resulting from high electric field treatment presented higher values of CI and TPI than controls, these values were lower than those of wines obtained after the low electric field treatment (Delsart et al., 2014). Although most of the studies related to mass transfer processes have been applied to red-winemaking, the potential application of PEF treatments has also been evaluated for the production of rosé wines.

Rosé wines are obtained from red grape varieties and the colour extraction, from crushed grapes, is attained by applying brief maceration times of crushed grapes and must before the fermentation process (Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2006). Maceration at temperatures lower than 20 °C improves the aromatic stability and the organoleptic characteristics of the final wines (Bisson, Daulny, & Bertrand, 1980). In view of the positive results obtained from PEF-assisted maceration-fermentation of red wines, the application of PEF treatments was evaluated by Puértolas et al. (2011) for production of rosé wines, derived from Cabernet sauvignon variety, at low maceration temperatures. PEF treatment consisted of 50 square wave pulses of 3 μs width at 122 Hz of frequency and electric field strength of 5 kV/cm. The treatment was applied in a continuous flow to respective mixtures of crushed grapes. The maceration processes were performed at temperatures between 4 and 20 °C and maceration times from 0 to 6 h. After the vinification, the different wines were stabilized and stored at 18 °C for 2 months. Wines from PEF-treated grape skins presented higher concentration of anthocyanins than control wines for all the conditions studied. It was also estimated that, to obtain the optimal concentration of anthocyanins (50 mg/L), the maceration time of PEF-treated samples at 4 °C was 2.7 h lower than for control samples at the same temperature. Furthermore, for control samples, to reach the same anthocyanin concentration in the same period as in PEF-treated samples, the temperature had to be increased up to 20 °C. Therefore, the authors suggested that the positive effects of PEF treatments could serve to reduce the standardized maceration process of rosé wines that in turn could be performed at much lower temperatures than the temperatures normally used. This can lead to a positive improvement on the organoleptic characteristics of rosé wines.

In addition to the phenolic extraction of grape skins promoted by PEF treatments, extraction of primary aromas from grape skins has been described in Garde-Cerdán et al. (2013). In this work, the influence of four different PEF treatments on the volatile composition of three grape varieties, Graciano, Tempranillo, and Garnacha, was studied. In order to do this, PEF treatments were carried out with crushed and destemmed grape harvest products (must, skin, seed) using a continuous flow system. The total average residence time in the treatment chamber was 0.09–0.10 s and the pulses applied were of 10 and 20 μs at 7.4 kV/cm, with frequencies of 300 or 400 Hz. The results showed that the influence of PEF treatments on the grape volatile composition depended on the variety. However, the flavour profile of the samples was not affected in any case. The content of terpenes decreased upon treatment in Graciano variety. In the case of Tempranillo, minor differences were observed for these compounds after PEF treatments. However, in the case of Garnacha the amount of terpenes was enhanced by the treatments, regardless of the PEF applied. As in the case of terpenes, lower energy treatments were detrimental to C_{13} norisoprenoids for Graciano. In the case of Tempranillo, in general terms, all treatments had a negative effect on the presence of these compounds, whereas for Garnacha a positive effect was observed in the amount of β-ionone. With regard to esters, the lower energy treatments favoured their presence in Graciano, intermediate energy treatments in Tempranillo, and medium-high energy treatments in

Garnacha. The content of benzenoid compounds was slightly affected by the treatments for Graciano and Tempranillo, while PEF had a positive effect on Garnacha. Finally, few differences were found between treatments for C6 compounds. Therefore, it can be concluded that the volatile composition of grape juice was enhanced by PEF application in Garnacha. However, no significant improvement was observed in Graciano and Tempranillo. To date, there has not been any other study for the evaluation of PEF treatments on the extraction of primary aroma compounds from grapes.

PEF treatments can also be applied on grapes to improve the pressing process. It has been shown that PEF-assisted pressing produces higher amount of must than traditional pressing (Grimi, Lebovka, Vorobiev, & Vaxelaire, 2009; Praporscic et al., 2007). Furthermore, the process is accelerated and consequently the risk of browning, characteristic of white must and derived wines, decreases (Praporscic et al., 2007). Mild and low electric field in the range from 0.4 to 0.75 kV/cm, with low energy inputs of 15–20 (kJ/kg), are enough to produce grape skin permeabilization and subsequent improvement of the pressing process (Grimi et al., 2009; Praporscic et al., 2007).

5.5 Conclusions and Future Trends

Pulsed electric field is a versatile technique that can be applied at almost all stages of the fermentative process. By applying PEF to the grape pomace mixture (must, skin, seed), the extraction of important compounds present in grape skins could be favoured, although this effect greatly depends on the grape variety. Therefore, the application of PEF treatments, with mass transfer purposes, can increase the quality of the final wines and also decrease the maceration periods, hence winery production cost can be reduced. Another potential application of PEF treatments is the sterilization of must and wines. Thus, PEF-sterilized must can be directly consumed or employed for wine production. In the case of must intended for direct consumption, PEF treatments decrease the microbial population without modifying the original composition of the product, thus the treatment has potential applications in the juice industry. In the case of the application of PEF to the must intended for vinification, the treatment not only reduces microorganism population without altering the composition of the must, but also inactivates naturally occurring enzymes responsible for oxidative reactions. Therefore, this technology can be employed as an alternative to the use of SO_2 or may permit the reduction of SO_2 addition before the vinification or the ageing process. However, more studies are necessary to evaluate the effects of PEF on wine composition, mainly regarding the volatile compounds. Although PEF is a promising technology that offers many advantages for wine production, its application is expensive due to the high cost of PEF devices and the high energy input that has to be applied, especially for microbial inactivation. This may explain why use of this technology is not widespread in food production and why it has not yet been implemented in the wine sector. Therefore, further studies intended

to improve PEF devices so as to increase the effectiveness of the treatment at reduced cost are necessary.

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Chapter 6
Ultrasound and Food Fermentation

K. Shikha Ojha, Colm P. O'Donnell, Joseph P. Kerry, and Brijesh K. Tiwari

6.1 Introduction

Food fermentation which has been reported since ancient times involves chemical transformation of complex organic compounds into simpler compounds by the action of enzymes and microorganisms including yeast, moulds and bacteria (Corma, Iborra, & Velty, 2007). Fermentation processes have been developed for the production of a wide range of products from chemically simple compounds, e.g. ethanol to highly complex macromolecules, e.g. polysaccharides. The modern fermentation industry is highly competitive and innovative, and has been at the forefront in assessing the potential of new technologies to improve fermentation processes and yield higher quality products. The literature suggests that novel technologies for food fermentation will assist food processors to meet both consumer demands for higher quality and safer products and the food industry demand for energy efficient processes (Pereira & Vicente, 2010). The food fermentation industry requires novel techniques to improve the productivity and quality of fermented products along with the new analytical tools to study and monitor complex fermentation processes. Various novel processing and monitoring technologies

K.S. Ojha (✉)
Food Biosciences, Teagasc Food Research Centre, Dublin 15, Ireland

School of Food & Nutritional Sciences, University College Cork, Cork, Ireland
e-mail: Shikha.ojha@teagasc.ie

C.P. O'Donnell
School of Biosystems and Food Engineering, University College Dublin, Dublin, Ireland

J.P. Kerry
Food Packaging Group, School of Food & Nutritional Sciences, University College Cork, Cork, Ireland

B.K. Tiwari
Food Biosciences, Teagasc Food Research Centre, Dublin 15, Ireland

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including ultrasound have been investigated to enhance the productivity and process efficiency of food fermentation.

Ultrasound is a versatile technology which is ideally suited to both processing and monitoring applications. Ultrasound is employed in various sectors including chemical, bioprocessing, food processing, pharmaceutical, medical and defence (Awad, Moharram, Shaltout, Asker, & Youssef, 2012; Chemat & Khan, 2011). Within the food industry, high frequency ultrasound is typically used as a non-destructive, non-invasive analytical technique for quality assurance, process monitoring and control, whereas low frequency sonication is employed for process intensification.

This chapter provides an overview of the theory and fundamentals of ultrasound technology. Potential applications of ultrasound in food fermentation and process monitoring applications are also discussed.

6.2 Fundamentals of Ultrasound

Ultrasonic waves are sound waves having frequencies above the human hearing range (>16 kHz). Ultrasonic waves can be classified based on frequency into three categories, namely: (1) power ultrasound (20–100 kHz); (2) high frequency or extended range for sonochemistry (20 kHz–2 MHz) and (3) diagnostic ultrasound (>1 MHz). Generally, from an application perspective, ultrasonics can also be broadly classified as low intensity (<1 W/cm^2) and high intensity (10–1000 W/cm^2) sonication. High frequency ultrasound employs low power levels which exert no or minimal physical and chemical alterations in the material through which the waves pass, hence it can be employed for food analysis and quality control applications. In contrast, the low frequency ultrasound employs higher power levels to induce desirable physical and chemical modifications for various bioprocessing applications.

Ultrasound is a form of vibrational energy produced by ultrasonic transducers which convert electrical energy into vibrational sound energy. Ultrasonic transducers are also capable of converting sound waves into electrical energy and are available in a range of size and frequencies depending on the application. Piezoelectric and magnetostrictive are the two most commonly used transducers (Mason & Peters, 2002). Various transducer types and generation of low or high frequency ultrasonic waves for a wide range of food and non-food applications have been reviewed extensively (Mattiat, 2013; Nakamura, 2012). However transducer types and their mechanisms of action are outside the scope of this chapter.

6.2.1 Low Frequency Ultrasound

Low frequency ultrasound generated using a transducer is transmitted using either an ultrasonic bath or probe based system operating at various frequencies. Placement of the transducer is important for even distribution of ultrasonic energy for process intensification. The major advantage of a bath system arrangement is that transducers are not in direct contact with the sample, but significant losses of acoustic energy occur to the vessel and surroundings. One of the most important aspects of the use of power ultrasound in food fermentation is the need to determine the optimum amount of acoustic energy to be applied. There are several methods to measure the energy input and one of the most commonly employed methods is calorimetry. Ultrasonic power, intensity and acoustic energy density can be calculated using the following equations (Eqs. 6.1–6.3).

$$\text{Power}\ (\text{W}) = mC_{\text{p}}\left[\frac{dT}{dt}\right]_{\tau=0} \tag{6.1}$$

$$\text{Ultrasonic intensity}\ \left(\text{W/cm}^2\right) = \frac{P}{A} \tag{6.2}$$

$$\text{Acoustic energy density}\ (\text{W/mL}) = \frac{P}{V} \tag{6.3}$$

where m is the mass, C_{p} is the specific heat capacity, A is the area of the radiating surface, V is the volume and (dT/dt) is the initial rate of change of temperature during sonication which can be determined by fitting the data obtained for temperature rise (using a standard thermocouple) against time to a polynomial curve and extrapolating to time $(t)=0$.

6.2.2 High Frequency Ultrasound

The basic principle of high frequency ultrasound employed for food process monitoring and analysis is based on absorption, reflection and transmittance of sound waves when passed through a medium. The velocity with which an ultrasound wave passes through a medium is dependent upon the medium properties, e.g. density and resistance offered by the medium to the propagating sound waves. Ultrasonic velocity, acoustic impedance and acoustic absorption are key ultrasound properties which enable characterisation and measurement of various physico-chemical properties of fermentation media. Ultrasonic velocity (c) is determined by the density (ρ) and elasticity (E) of the medium (Eq. 6.4). Acoustic impedance is determined by the density and ultrasonic velocity (Eq. 6.5), whereas acoustic absorption in a medium is influenced by the sound frequency and viscosity of the medium (Eq. 6.6). In the

case of complex solutions including non-homogenous solutions containing bubbles or insoluble particles, acoustic absorption is also influenced by thermal relaxation and scattering losses

$$\text{Ultrasonic velocity } (c) = \sqrt{\frac{E}{\rho}} \tag{6.4}$$

$$\text{Acoustic impedence } (Z) = \rho \cdot c \tag{6.5}$$

$$\text{Acoustic absorption } (\alpha) = \frac{2\pi^2 \cdot f^2}{\rho \cdot c^3}\left(\frac{4}{3}\eta_s + \eta_v\right) \tag{6.6}$$

η_v and η_s are the bulk and shear viscosity, f is the frequency

$$\text{Acoustic absorption } (\alpha) = \alpha_{\text{viscous}} + \alpha_{\text{thermal}} + \alpha_{\text{relaxation}} + \alpha_{\text{scattering}} \tag{6.7}$$

The changes in key acoustic properties along with advanced signal processing techniques facilitate continuous control and monitoring of fermentation processes (Schäfer, Carlson, & Hauptmann, 2006). Determination of acoustic impedance and absorption is challenging; however, precise measurement of velocity is possible by measuring the time of flight via a known constant sound path (Henning & Rautenberg, 2006).

Pulse-echo and continuous wave ultrasound are the two most commonly employed techniques in ultrasound sensors for monitoring applications. The pulse-echo technique requires a transducer and oscilloscope and is the most popular method for the determination of acoustic velocity and attenuation in solids and liquids (Pal, 2015). In this technique an ultrasonic transducer is attached to a fermentation vessel which transmits a signal through the fermentation chamber containing the sample which is reflected back to the transducer after hitting the fermentation chamber wall (Fig. 6.1). The continuous wave technique (also known as through transmission) employs either two transducers one for emitting and another for receiving signals or one single transducer (dual element transducer) capable of continuously receiving and emitting signals. A dual element transducer is useful for thickness measurement of thin materials and for measurement of surface properties (Mitri, Kinnick, Greenleaf, & Fatemi, 2009). In the continuous wave technique, one transducer converts electric pulses to ultrasonic waves which are transmitted through a chamber while the second transducer converts the ultrasonic signal transmitting through the sample to electric signals which are recorded by oscilloscope. The signals transmitted through the sample can be used to monitor concentrations and biochemical transformations occurring within the fermentation chamber using key ultrasonic properties.

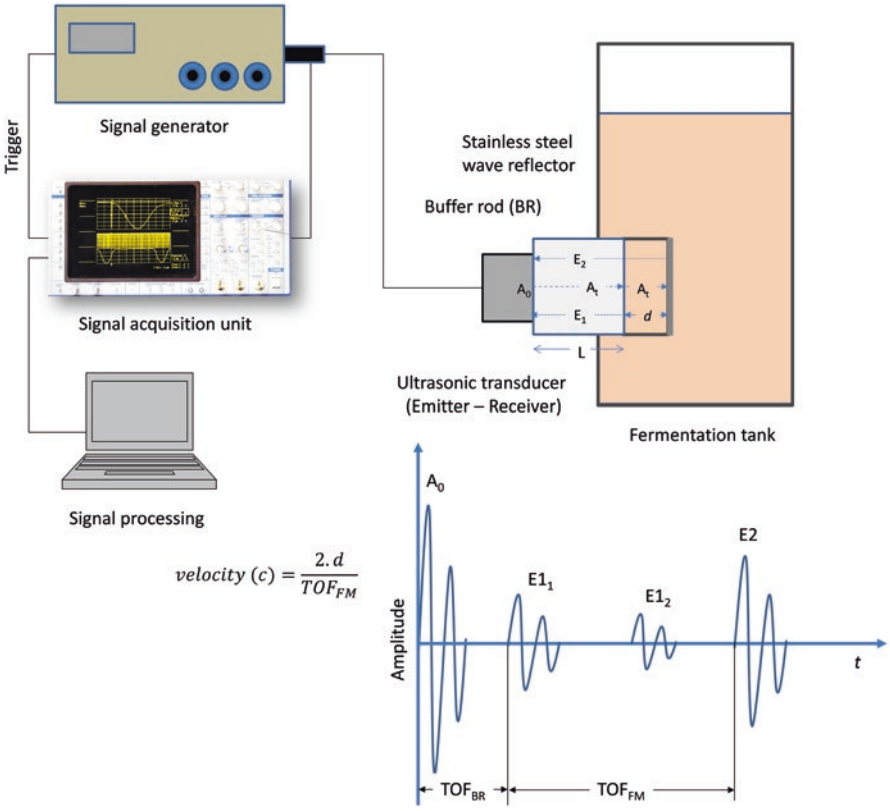

Fig. 6.1 Ultrasound based set-up in a fermentation tank for monitoring of fermentation process (modified from Novoa-Díaz et al., 2014)

6.2.3 *Physical and Chemical Effects of Ultrasound*

Physical and chemical phenomena of ultrasound associated with ultrasound frequencies include agitation, vibration, pressure, shock waves, shear forces, microjets, compression and rarefaction, acoustic streaming, cavitation and formation of free radicals. The physical effects of ultrasound are dominant in the lower frequency range of 20–100 kHz with a higher level of transient cavitation, whereas chemical effects are dominant in range of 200–500 kHz due to generation of large number of active bubbles (Feng, Barbosa-Cánovas, & Weiss, 2011). Acoustic streaming is dominant at higher frequencies (>1 MHz) with less physical and chemical effects associated with cavitation. Acoustic streaming is a physical force of the sound due to a pressure gradient which is capable of displacing ions and small molecules. Cavitation or Blake threshold is defined as the lowest acoustic pressure at which bubble formation is observed. The phenomenon of the creation, expansion and implosive collapse of microbubbles in ultrasonically irradiated liquids is known as

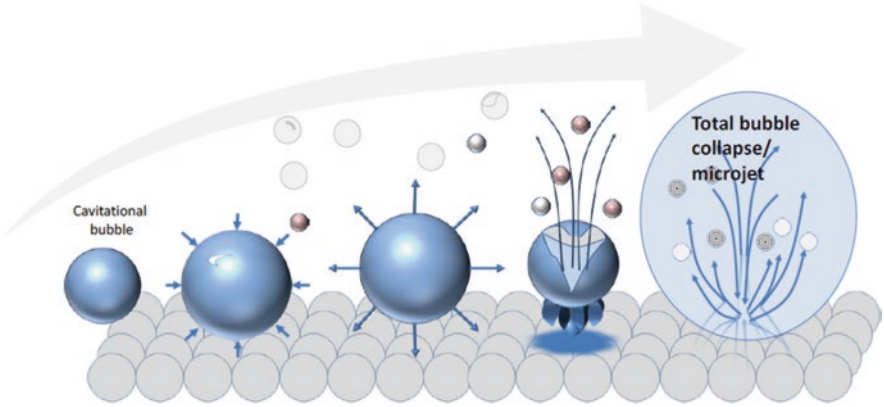

Fig. 6.2 Formation and collapse of cavitation

"acoustic cavitation" (Fig. 6.2). At higher frequencies acoustic pressures are lower and hence cavitation rarely occurs, whereas at low frequencies, ultrasound waves of high acoustic pressures are possible, hence cavitation is observed.

The effectiveness of ultrasound on microorganisms and enzymes employed for fermentation is strongly influenced by various factors including microbial ecology (e.g. type of microorganism, medium type and composition), ultrasound parameters (e.g. ultrasound power and frequency), sonication time, pH and temperature (Moncada, Aryana, & Boeneke, 2012). Studies have shown that ultrasound can inactivate microorganisms. However inactivation of microorganisms occurs at low frequencies, whereas when microbial cells are exposed to a higher frequency range microbial cells are minimally affected with no significant effect on cell viability. For example, Radel, McLoughlin, Gherardini, Doblhoff-Dier, and Benes (2000) did not observe any significant changes in the viability of yeast cells even though some rearrangement of cellular components was reported when subjected to ultrasonic frequency at 2.2 MHz. Rearrangement of cellular components was principally due to damage of vacuoles within the cell while the nucleus and cell wall were unaffected. Inactivation of microorganisms using low frequency sonication is due to various chemical and physical effects including localised heating, intracellular cavitation, acoustic streaming, micromechanical shock waves, and sonolysis of water ($H_2O \rightarrow H^+ + OH^-$) leading to the production of free radicals (O'Donnell, Tiwari, Bourke, & Cullen, 2010; Shirsath, Sonawane, & Gogate, 2012). Physical and chemical effects owing to ultrasound induce thinning/disruption of cell membranes leading to microbial inactivation. The effect of ultrasound is also dependent on the type of microorganism. For example, studies have shown that Gram-positive bacteria are more resistant to ultrasound compared to Gram-negative bacteria, possibly because Gram-positive bacterial cells possess a thick and more robust cell wall due to cross-linking of peptidoglycan and teichoic acid (Monsen, Lövgren, Widerström, & Wallinder, 2009).

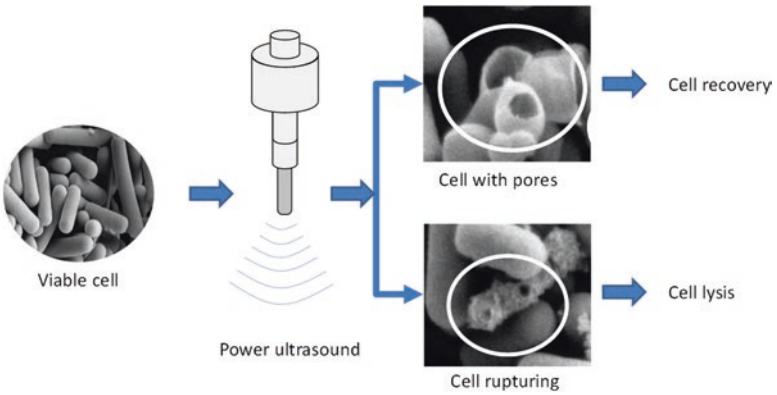

Fig. 6.3 Effect of ultrasound on probiotics

Ultrasound has been demonstrated to enhance the growth of beneficial microorganisms and activity of enzymes, resulting in the production of biologically active macromolecules. The beneficial effects of sonication are mainly attributed to the formation of pores on microbial cell membranes, thereby providing a channel for transport of essential nutrients and removal of toxic substances across these membranes (Yang et al., 2008; Yeo & Liong, 2012, 2013). Microscopic images have shown that ultrasound induces pits or holes leading to a microbial cell injury due to sonoporation. Sonoporation can be defined as the formation of temporary holes in the cell membrane. It is thought to be a temporary phenomenon which could improve the permeability of the cell membrane (Lentacker, De Cock, Deckers, De Smedt, & Moonen, 2014). However an increase in ultrasonic power or exposure time can lead to inactivation or cell death due to leakage of cellular content as shown in Fig. 6.3. The effect of ultrasound on enzymatic activity is enzyme specific and the effect of ultrasound on various enzymes was reviewed by O'Donnell et al. (2010). Stimulation and/or retardation of enzymatic activity due to sonication is not well understood. However the influence of ultrasound on enzymes can be attributed to changes in protein confirmation, controlled denaturation or accelerated collision probability of the enzyme and substrate (Mason, Paniwnyk, & Lorimer, 1996; Szabó & Csiszár, 2013; Wang et al., 2012).

6.3 Application of Ultrasound in Fermentation

Application of both low and high frequency ultrasound to fermentation processes industry has been widely investigated. High frequency ultrasound is typically used as a non-destructive analytical technique for monitoring fermentation processes (Novoa-Díaz et al., 2014), whereas low frequency ultrasound has been employed to enhance fermentation rates (Masuzawa, Kimura, & Ohdaira, 2003; Nguyen, Lee, & Zhou, 2012),

pasteurisation (Gracin et al., 2015) and other specialised processing applications including wine maturation and ageing (Tao, García, & Sun, 2014), degassing or deaeration of alcoholic beverages (Chemat & Khan, 2011; Matsuura, Hirotsune, Nunokawa, Satoh, & Honda, 1994). The main applications are discussed below.

6.3.1 Application of Power Ultrasound

Numerous applications of power ultrasound in food processing have been reported. However applications in food fermentation are mainly for improving enzyme/microorganism performance, foam destruction, emulsification and improving end product quality and safety. Application of low frequency ultrasound for various fermentation applications is outlined in Table 6.1. The main applications of ultrasound in key sectors are discussed below. Application of low frequency (20–100 kHz) ultrasound alone or in combination with heat and/or pressure for improving the safety profile of milk has been reported to achieve the desired 5 log reduction of pathogenic microorganisms including *Listeria innocua* and *Escherichia coli* (Bermúdez-Aguirre, Corradini, Mawson, & Barbosa-Cánovas, 2009; Lee, Zhou, Liang, Feng, & Martin, 2009). Low frequency ultrasound processing of milk is also reported to induce desired physico-chemical changes in macromolecules including enzyme modification, homogenisation, pasteurisation, reduction in yoghurt fermentation time (Wu, Hulbert, & Mount, 2000) and improved rheological properties of yoghurt (Vercet, Oria, Marquina, Crelier, & Lopez-Buesa, 2002). Studies have shown that ultrasound processing of milk offers potential to achieve pasteurisation and homogenisation effects whilst reducing yoghurt production time (up to 40 %) with significant improvement in rheological properties (e.g. consistency and texture) of the final product (Dolatowski, Stadnik, & Stasiak, 2007). Sonication of milk prior to inoculation of starter culture increases water holding capacity and viscosity and decreases syneresis, whereas sonication treatment after inoculation has been shown to have no beneficial effect on syneresis while reducing fermentation time by 30 min (Wu et al., 2000). Improved water holding capacity could be due to ultrasound induced homogenisation effect which causes a change in water holding capacity of the milk proteins and tends to reduce syneresis. In another study, Riener, Noci, Cronin, Morgan, and Lyng (2010) also observed a significant reduction in syneresis levels of gels prepared from thermosonicated milk compared to conventional counterparts. Mild manothermosonication, i.e. application of heat and ultrasound (117 μm amplitude, 20 kHz frequency) under moderate pressure (2 kg/cm^2) treatment of milk prior to yoghurt preparation was reported to have improved structural properties compared to conventionally processed yoghurt (Vercet et al., 2002). Improved structural properties could be attributed to ultrasound effects on the fat globule membrane which would modify the ability of fat globules to interact among themselves and also with milk protein (casein) micelles. Reduction in fat globule size as a result of sonication cannot be considered as a factor alone for

Table 6.1 Application of low and high frequency ultrasound in food fermentation

Products	Ultrasonic conditions	Salient findings	References
Low frequency ultrasound			
Full-fat yoghurt	20 kHz for 1–10 min, before or after culture inoculation	Higher water holding capacity	Hongyu, Hulbert, & Mount (2000)
		Higher viscosity	
		Lower syneresis	
		Reduction in fermentation time by 30 min	
Sweet whey	Microbial culture activation at 84 W for 150 s	Reduced fermentation time	Barukčić, Jakopović, Herceg, Karlović, and Božanić (2015)
		Higher viable counts	
Probiotic fermented milk	20 kHz for 7–30 min at power level of 100 W	Improved fermentation rates	Nguyen, Lee, and Zhou (2009), Nguyen et al. (2012)
		Accelerates lactose hydrolysis	
		Stimulation of bifidobacteria	
		High level of oligosaccharides	
Rice wine	20 kHz ultrasound for 1 week	Alcohol content reduction	Chang and Chen (2002)
		Acetaldehyde content decrease	
		Ethyl acetate content increase	
		Polyol concentration reduction	
Red wine	26 kHz ultrasound (118 W) for 20 min	Cell viability of spoilage microorganisms in wine decreased	Luo, Schmid, Grbin, and Jiranek (2012)
		Modification of wine flavour and aroma profile	
Model wine used was a 12% (v/v) aqueous ethanol solution, acidulated to pH 3.5 with tartaric acid.	25 kHz ultrasound (at acoustic energy density of 6.3, 14.9 and 25.8 W/L) and temperature (15, 20, 25 °C)	Total phenolic yield released was not affected by acoustic energy density significantly	Tao, García, et al. (2014), Tao, Zhang, and Sun (2014)
		Total phenolic yield increased with the increase of temperature during sonication	
Model wine composed of a water–alcohol solution in ratio of 9:1 (v/v), acidulated to pH 3.5 with tartaric acid. 5 g/L dry lees were added	40 kHz ultrasonic bath system in pulse mode of 1 on and 1 h off for exposure time of 1, 2, 3, 10, 15, 23.5 and 48 h	Ultrasound treatment markedly increased the release of proteins	Martín, Guillemet, Feng, & Sun (2013)
		Viability of the yeast was significantly affected by ultrasound: after 20 h ultrasonic treatment	

(continued)

Table 6.1 (continued)

Products	Ultrasonic conditions	Salient findings	References
Red wine	Ultrasonic processor (400 W, 24 kHz, 100 μm amplitude) in continuous flow treatment at 30 and 40 °C	Reduction of *Brettanomyces* (89.1–99.7 %) and lactic acid bacteria (LAB) (71.8–99.3 %) impaired aroma of wine due to formation of negative oxidative smell	Gracin et al. (2016)
Red wine and model medium composed of water/ethanol (90:10 mL/mL) acidulated to pH 3.5 with tartaric acid	50 min per week with an ultrasound equipment having a frequency of 50 KHz	Significant increase in the concentration of polysaccharides released into the wine after only 2 weeks overall depletion in the anthocyanin content	Kulkarni, Loira, Morata, Tesfaye, González, & Suárez-Lepe (2015)
		Oxidative taste in sensory analysis	
Red wine and the model wine	100 kHz ultrasound (300 W power) for 5 min at 20 °C	Increase in the intensity of DMPO/1-hydroxylethyl free radical	Zhang, Shen, Fan, & García Martín (2015)
High frequency ultrasound			
Alcoholic fermentation	Frequency: 2 MHz, measurement of sound velocity	Density and ultrasonic velocity in the ternary mixture (water–ethanol–sucrose)	Resa, Elvira, Montero de Espinosa, and Gómez-Ullate (2005)
Wine fermentation	Frequency: 1 MHz, measurement of sound velocity	Monitoring of malolactic fermentation process	Novoa-Díaz et al. (2014)
Yoghurt fermentation	Frequency: 3.7 MHz, measurement of sound velocity, phase difference of acoustic wave	Monitoring of phase change from liquid to gel	Ogasawara, Mizutani, Ohbuchi, and Nakamura (2006)
Malolactic fermentation in wines	Frequency: 1 MHz; measurement of sound velocity	Predict the end point of the malolactic fermentation process; malic and lactic acid concentrations	Amer, Novoa-Díaz, Puig-Pujol, Capdevila, Chávez, Turó, et al. (2015)
Beer fermentation	Frequency: 2 MHz; measurement of sound velocity	Ternary system water–maltose–ethanol with respect to density, speed of sound and temperature (5–30 °C)	Hoche, Hussein, & Becker (2015)
Model fermentation	Ultrasonic velocity and attenuation measurement	Simultaneously determining yeast and maltose concentration	Geier, Heermann, Hussein, & Becker (2014)
	Ultrasonic frequency: 2 MHz		
Dough fermentation	Ultrasonic velocity and attenuation measurement	Measurement of dough density as an indication of fermentation time	Elmehdi, Page, and Scanlon (2003)
	Ultrasonic frequency: 50 MHz		

improved textural properties because improved texture was not observed in yoghurt prepared from conventionally homogenised milk samples (Vercet et al., 2002). In a similar study, Riener, Noci, Cronin, Morgan, and Lyng (2009) showed that yoghurt prepared from thermosonicated milk (24 kHz for 10 min) with varying levels of fat (0.1, 1.5 and 3.5 %) had higher viscosities and water holding capacities compared to those prepared from conventionally heat-treated milk (90 °C for 10 min). Yoghurt prepared from theromosonicated milk was shown to possess a honeycomb like network exhibiting large number of pores throughout the structure with small particle size (<1 μm) compared to conventional yoghurt which showed a dense structure. Thermosonication (20 kHz, 480 W/55 °C for 8 min) treatment of reconstituted whey powder has been shown to increase the viable count of dairy starter culture at the end of fermentation time with improved organoleptic properties compared to thermally processed samples (Barukčić et al., 2015). A reduction in fermentation time while increasing lactose hydrolysis and improving acidifying activity of probiotics has been demonstrated in numerous studies. Nguyen et al. (2009) and Nguyen et al. (2012) demonstrated the potential of low frequency ultrasound in the stimulation of probiotics (e.g. *Bifidobacterium* sp.) resulting in accelerated lactose hydrolysis and transgalactosylation of bifidobacteria in milk while reducing fermentation time by up to 30 min depending on probiotic strain. Nguyen et al. (2009) observed an initial decrease in probiotic cell count at the beginning of fermentation time compared to control with no significant changes in the final counts at the end of fermentation. An increase in viability of probiotics by up to 0.49–0.57 $\log_{10}$ CFU/mL and 0.26–0.57 $\log_{10}$ CFU/mL has been reported for *Lactobacillus* sp. and *Bifidobacterium* sp. compared to the control in the case of fermented soy milk (Yeo & Liong, 2011). In another study, Barukčić et al. (2015) investigated ultrasonic activation of monoculture (*Lactobacillus acidophilus*, La-5) and mixed culture (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. Bulgaricus, YC-380). They observed that the ultrasonic activation of La-5 inoculum did not influence the viable cells count regardless of the applied conditions compared to the untreated inoculum. However, ultrasonic activation of YC-380 at 84 W for 150 s resulted in approximately 1 log cycle higher count compared to untreated inoculum (activated at 37 °C/30 min) with a decrease in fermentation time by up to 30 min. Probiotic cells treated with ultrasound have been shown to recover from injury and subsequent increase in number during fermentation depending on the microorganisms and ultrasound processing conditions. These results demonstrate that the effect of sonication is culture specific, depending on distinctive resistance of microorganisms towards ultrasound due to variations in cell wall thickness, composition and cell size. Application of ultrasound during fermentation has also been shown to improve β-galactosidase activity of probiotics resulting in the production of health promoting oligosaccharides in fermented milk and improvement of isoflavones bioconversion activities in soymilk. In some cases inoculation of injured probiotics (*bifidobacteria* and *Lactobacillus*) has been shown to result in improved viability and stability during fermentation of yoghurt (Shah & Lankaputhra, 1997). The effect of ultrasound on probiotics is not well understood.

Application of low frequency ultrasound in the wine industry is employed for managing wine microbiology (Jiranek, Grbin, Yap, Barnes, & Bates, 2008; Luo et al., 2012), extraction of volatile compounds from wine (Cabredo-Pinillos, Cedrón-Fernández, González-Briongos, Puente-Pascual, & Sáenz-Barrio, 2006), improving fermentation rates (Matsuura et al., 1994), wine ageing and maturation (Chang & Chen, 2002). Reduction in fermentation time at low ultrasonic intensities has been shown to accelerate the growth of *S. cerevisiae* (Lanchun et al., 2003) and *Kluyveromyces marxianus* (Sulaiman, Ajit, Yunus, & Chisti, 2011) depending on the ultrasonic processing parameters. A 50–64 % reduction in fermentation time has been reported for wine, beer and sake obtained from saccharified rice solution at ultrasonic intensity of 30 mW/cm^2 operating at 43 kHz (Matsuura et al., 1994). Ultrasound has been shown to improve yeast performance without affecting cell viability via morphological changes in yeast cells. Jomdecha and Prateepasen (2011) observed a significant changes in the lag phase of yeast cells depending on ultrasonic intensities/energies and treatment time. They observed that the ultrasonic energies operating at a frequency of 20 kHz in a range of 330 and 360 W s/m^3 could decrease lag time by up to 1 h compared to control, whereas ultrasonic energy >850 W s/m^3 could increase the lag time resulting in reduced growth. The potential of ultrasound to accelerate wine ageing and maturation thereby improving wine quality has been reported extensively (Martín & Sun, 2013; Tao, García, et al., 2014). Effect of ultrasound on wine ageing and maturation has shown varying degree of ageing effect depending on ultrasonic conditions and wine type. Chang and Chen (2002) investigated the effect of 20 kHz ultrasound on wine ageing prepared from rice and maize. They observed an enhanced ageing effect on rice wine due to sonication with sensorial attributes similar to that of conventionally aged wine while no effect on ageing of maize wine was reported. Ultrasound technology has been demonstrated to enhance the release of oak-related compounds from oak chips into wine during wine ageing with oak chips. A significant increase in total phenolic content in model wine was reported during ultrasound treatment for 150 min at 25 kHz depending on acoustic energy density and temperature (Tao, Zhang, et al., 2014). Ultrasound has also been demonstrated as an alternative technique to inactivate spoilage microorganisms associated with wine. Currently use of chemical preservatives (e.g. sulphur dioxide, dimethyl dicarbonate), thermal pasteurisation or removal of spoilage microorganisms by filtration are commonly employed techniques for improving the shelf-life of wine (Bartowsky, 2009; Du Toit & Pretorius, 2000). In a study, Luo et al. (2012) investigated the effect of ultrasound (24 kHz) for 20 min on various types of yeast (*Dekkera bruxellensis, Hanseniaspora uvarum*, *Pichia membranefaciens*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Zygosaccharomyces bailii*) and bacteria (*Acetobacter aceti*, *Acetobacter pasteurianus*, , *Oenococcus oeni*, *Pediococcus sp.*) associated with wine. They observed that the viability of yeast was more affected compared to the spoilage bacteria investigated. In another study, Gracin et al. (2016) employed continuous flow through high power sonication (400 W, 24 kHz, 100 μm amplitude) to reduce spoilage microorganisms. They observed a significant reduction in microbial counts of *Brettanomyces* (89.1–99.7 %) and lactic acid bacteria

(71.8–99.3 %) in wine. However, the ultrasound caused negative changes in wine sensorial properties with the formation of negative oxidative smell of burns or smoke and oxidised aroma.

6.3.2 Application of High Frequency Ultrasound

High frequency ultrasound has been employed in many industrial processes for several decades. Ultrasonics has been demonstrated as a potential tool for non-invasive, non-destructive, rapid and precise measurements of various parameters, for example, volume measurement of agriculture products (Nishizu, Torikata, Yoshioka, & Ikeda, 2005), online measurement of concentrations in solutions (Masuzawa et al., 2003), food composition, structure, flow rate, physical state and molecular properties (McClements & Gunasekaran, 1997). Studies have shown that acoustic based methods can be employed in measurement systems that are non-invasive, hygienic, precise, rapid, low cost and suitable for automation (Novoa-Díaz et al., 2014; Stillhart & Kuentz, 2012). Table 6.1 lists examples of high frequency ultrasound employed for monitoring fermentation processes.

Traditionally, fermentation processes are monitored or controlled by withdrawing samples at regular intervals for estimation of key fermentation parameters including microbial growth, pH, acidity, turbidity and chemical composition. Determination of fermentation process parameters by classical chemical analysis is time consuming and does not facilitate real time control. Application of high frequency ultrasonic waves can provide useful information to characterise fermentation processes involving homogenous and/or multiphase systems, with no degradation or chemical alterations reported in fermentation media due to sonic waves (Henning & Rautenberg, 2006). The ultrasonic velocity of an ultrasonic wave travelling through a fermentation tank can be used to infer the concentration of alcohol and other sugars during the fermentation process (Resa et al., 2005). Studies have shown an empirical relationship between ultrasonic parameters and the concentration of alcohol and soluble solids in wine (Winder, Aulik, & Rice, 1970) and density of beer (Becker, Mitzscherling, & Delgado, 2001) during fermentation.

Several ultrasonic parameters including pulse-echo or transmission-through mode, power attenuation, measurement of velocity by time of flight, indirect prediction of acoustic impedance via reflection coefficient have been employed for estimation of fluid density and other parameters in various liquid, semi-liquid and multiphasic systems (Bamberger & Greenwood, 2004; Kuo, Sheng, & Ting, 2008). For example, Novoa-Díaz et al. (2014) reported that a change in ultrasonic velocity is strongly correlated to the concentration of malic acid and lactic acid during red wine fermentation due to the action of lactic acid bacteria. They observed an increase in ultrasonic velocity with an increase in lactic acid concentration and a decrease in velocity due to an increase in malic acid concentration. Krause, Hussein, Hussein, and Becker (2014) developed a multivariate regression method to predict maltose concentration in aqueous solutions at different temperatures by utilising the

time and frequency domain of ultrasonic signals. They reported that partial least square regression models are least influenced by bubbles and other suspended particles during fermentation.

Apart from measuring the concentration of chemical compounds during fermentation process, high frequency ultrasound (>15 MHz) has been demonstrated to measure the concentration of yeast cells in liquid suspensions. A backscattering ultrasound based technique with an ultrasonic emitter/receiver wideband focused transducer centred at 75 MHz showed improved sensitivity to detect yeasts at a concentration as low as 10^4 cells/mL (Elvira, Vera, Cañadas, Shukla, & Montero, 2016). The pulse spectrum reaching the transducer after backscatter by a yeast cell depends on the size and nature of the cell, the attenuation of the medium and the sound wave.

Changes in ultrasonic velocity can also be attributed to changes in temperature, density and pressure. In the case of yoghurt fermentation, Ogasawara et al. (2006) employed non-contact acoustic monitoring using a pair of acoustic transducers operating at a frequency of 3.7 MHz to determine the end point of a yoghurt production process. They correlated a phase difference between input and output signals measured by an oscilloscope to a phase change from liquid (milk) to gel (yoghurt) with an inflection point around 18 h indicating the end of the yoghurt fermentation process. In a dough fermentation study, Elmehdi et al. (2003) observed a relationship between ultrasonic wave parameters and dough fermentation characteristics. They observed a decrease in ultrasonic velocity and an increase in attenuation with fermentation time. They reported that a change in attenuation was correlated to the change in the dough void fraction during fermentation.

6.4 Conclusions and Future Outlook

In food fermentation processes, ultrasound technology has been employed both as a processing tool and for monitoring, analysis and control of fermentation processes. Several potential applications of low and high frequency ultrasound have been reviewed and these applications clearly demonstrate the potential of ultrasound in the food fermentation applications. However, there are various technological constraints which have an impact on industrial adoption and commercial viability of ultrasonic systems. Ultrasound processing to achieve desired physical and chemical effects is an energy intensive process. Scale-up issues are also a challenge.

A high level of energy consumption can be justified if the cost–benefit analysis of ultrasound is demonstrated. Future research should be focused on the sustainability of ultrasound assisted processes and process configuration to improve safety, quality and shelf-life profile of fermented food products while minimising the associated energy consumption. Mechanisms of microbial/enzyme stimulation and/or inactivation have been a subject of scientific interest. However, the interaction between ultrasound and microorganisms is complex and is not well established. Understanding key underlying mechanisms of action will allow greater understanding of processes and thus can assist in process scale-up. Nevertheless, ultrasound has been widely demonstrated to be an excellent technology which can be employed to enhance fermentation processes.

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Chapter 7
Gamma Irradiation and Fermentation

Mohamed Koubaa, Sonia Barba-Orellana, Elena Roselló-Soto, and Francisco J. Barba

7.1 Introduction

The four major types of radiation sterilization described in literature are γ-irradiation, e-beam, natural light, and microwave (Yaman, 2001). Sterilization by γ-irradiation has been widely used to inactivate microorganisms in food and pharmaceutical sectors. There are multiple advantages of γ-irradiation over other competitive techniques which include: its high penetration power, isothermal character, and the absence of residues. Sterilization by γ-irradiation is based on killing microorganisms by breaking their chemical bonds, producing free radicals that attack the nucleic acid of the microorganism, thus preventing its cellular division (Hasanain, Guenther, Mullett, & Craven, 2014). Gamma rays, measured in kiloGrays (kGy), present a radiopasteurization and radiosterilization technique for food preservation (Antonio et al., 2012). In fact, nowadays, food irradiation is increasingly recognized as a method for reducing postharvest food losses, ensuring hygienic quality, and facilitating wider trade in foodstuffs (Sadecka, 2010). Besides the food safety purposes, γ-rays treatment contributes to the preservation of the nutritional value of food products by protecting the thermolabile compounds from degradation due to nonthermal character of the

M. Koubaa
Sorbonne Universités, Université de Technologie de Compiègne, Laboratoire Transformations Intégrées de la Matière Renouvelable (UTC/ESCOM, EA 4297 TIMR), Centre de Recherche de Royallieu, CS 60319, Compiègne Cedex 60203, France

S. Barba-Orellana
Centro Sanitario Integrado de Xirivella, Consorci Hospital General Universitari València, C/Virgen de Los Desamparados, s/n 46950, Xirivella, Valencia, Spain

E. Roselló-Soto • F.J. Barba (✉)
Faculty of Pharmacy, Nutrition and Food Science Area, Universitat de València, Avda. Vicent Andrés Estellés, s/n. 46100, Burjassot, Spain
e-mail: francisco.barba@uv.es

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technique (Molins, 2001). Nevertheless, minor changes of food composition by generating free radicals can occur. Moreover, γ-irradiation is recognized to enhance alcohol production through fermentation and reduction of energy consumption. This chapter summarizes the impact of γ-irradiation on food composition, its application to preserve the integrity of food products by their radiosterilization and radiopasteurization along with their role in fermentation processes, which are also discussed.

7.2 Effects of γ-Irradiation

The direct action of γ-rays is mainly by damaging DNA, with its action on sugar base pairs whereas indirect effects are mainly due to the generation of ions, free radicals, and other reactive species. γ-Irradiation involves the chemical breakdown of the major food constituents as a result of either primary ions decomposition or primary ions reaction with neighboring molecules. When γ-irradiation is carried out on dried, frozen, or food containing solid particles, the generated free radicals upon irradiation have longer lifetime, due to their limited mobility, compared to $<10^{-3}$ s for fluid samples (Stewart, 2001). The generated free radicals may involve nutritional changes of food products by affecting their composition. The major constituents being affected are water, lipids, proteins, carbohydrates, and vitamins. Nevertheless, food irradiation can also degrade certain undesirable compounds (e.g. mycotoxins) present in the food.

7.2.1 Radiolysis of Water

Radiolysis of water contributes to the indirect effects of γ-irradiation by generating various reactive species. The main species formed during water irradiation by γ-rays are presented in reaction (7.1) (Simic, 1983).

$$H_2O \rightarrow e_{aq}^{-}(2.8) + {}^{\bullet}OH(2.8) + {}^{\bullet}H(0.5) + H_3O^{+}(2.8) + H_2(0.4)(6.1) + H_2O_2(0.8) \tag{7.1}$$

where e_{aq}^{-}, OH, H, H_2, H_2O_2, and H_3O^+ represent hydrated electron, hydroxyl radical, hydrogen atom (ion), hydrogen, hydrogen peroxide, and hydrated proton, respectively. The numbers between brackets represent the amounts of produced species per 100 eV absorbed, expressed as G-value. These species, especially free radicals, may react with various food components.

7.2.2 Radiation Chemistry of Lipids

Food lipids are composed of fatty acids and their derivatives (mono-, di-, triglycerides and phospholipids), with predominance of triglycerides. γ-Irradiation of these molecules leads to the fragmentation of fatty acids and glycerol backbones,

preferentially at –C–C– and –C– O– bonds. The same phenomenon is happening for free fatty acids with generation of species with double bonds for unsaturated fatty acids. The free radicals formed during γ-irradiation may react with each other, forming recombined products (Xuetong, 2012). In general, fatty acids generate higher fragments compared to triglycerides when subjected to γ-irradiation (Nawar, 1977).

Dietary intake of both saturated and *trans* fatty acids has been associated with an increase of coronary heart disease's risk (Ganguly & Pierce, 2012; Woodside, McKinley, & Young, 2008). To overcome these problems, the health organizations have recommended minimizing the consumption of food products containing these fatty acids. *Trans* fatty acids are present in minor quantities in vegetable oils, but their presence increases in processed foods as part of hydrogenation or partially hydrogenation process, and their addition as ingredients. Moreover, *trans* fat are naturally present in some meat products (e.g. beef and lamb), as a result of biohydrogenation (Xuetong, 2012). The formation of *trans* fat during irradiation of various food products was studied by Luck and Kohn (1963). The formation of *trans* fatty acids upon irradiation of ground beef was studied by Yılmaz and Geçgel (2007). The authors irradiated ground beef with γ-rays (0, 1, 3, 5, and 7 kGy) from a ^{60}Co source, and evaluated the impact on the formation of *trans* fatty acids. They demonstrated that irradiated samples had higher concentrations of total *trans* fatty acids compared to control samples, with highest total of *trans* fatty acids observed at 7 kGy. The amount of the formed *trans* fatty acids was proportional to the irradiation dose. Although the formation of *trans* fatty acids upon irradiation, many authors reported that the amount formed is less noticed than that occurring naturally (Fan & Kays, 2009; Xuetong, 2012). For example, commercial nonirradiated Japanese cattle beef showed a *trans* fatty acid range from 1.9 to 6.8 % of the total fatty acid content (Matsuzaki et al., 1998). The effect of γ-irradiation might be thus considered as negligible on *trans* fat formation.

7.2.3 Radiation Chemistry of Proteins

Free radicals generated by water radiolysis may react with proteins and amino acids generating a range of compounds. For example, when e_{aq}^{-} react with proteins, radiolytic compounds were found in irradiated foods, mainly produced by the deamination and scission of peptide and disulfide bonds (Delincee, 1983). Moreover, ·OH radicals may react with proteins, leading to the oxidation of some compounds following the abstraction of·H atom and its reaction with amino acids, especially sulfur-containing and aromatic residues (Stewart, 2001). Previous works demonstrated that these radiolytic products may decrease in frozen foods due to minor mobility of OH radicals (Nawar, 1977). γ-Irradiation of proteins is very similar to that occurring for amino acids. It results in several processes (e.g. deamination, decarboxylation, disulfide bonds reduction, sulfydryl groups oxidation, peptide-chains cleavage, and protein aggregation) (Delincee, 1983). The reaction mechanisms in the radiolysis of peptides, polypeptides, and proteins were reviewed by Garrison (1987).

The effect of γ-irradiation on the molecular properties of bovine serum albumin (BSA) was studied by Gaber (2005). The author investigated upon irradiation of BSA the effect of oxygen radicals on its molecular properties. The irradiation of BSA at various doses of γ-rays was followed by the study of the secondary and tertiary structures, molecular weight, and optical anisotropy. The author reported not only the disruption of the ordered structure of BSA (transformation from β-turns into β-sheets), but also its degradation (decrease of the molecular weight) and aggregation (through ultraviolet absorption spectroscopy measurements).

The occurring changes in proteins upon irradiation may impact the physical properties of the food product. In fact, the influence of γ-irradiation on physical properties of milk proteins was investigated by Cieśla, Salmieri, Lacroix, and Le Tien (2004). The authors found that γ-irradiation is an effective method to improve both barrier and mechanical properties of edible films and coatings based on calcium and sodium caseinates. They observed that the irradiated and heated samples showed higher content of β-strands, compared to heated control samples. A better-organized β conformation was noticed upon irradiation, compared to thermal treatment alone. These well-ordered β-strands improved the "crystalline" film properties, leading thus to an improved barrier characteristics and mechanical resistance as well as higher rigidity.

7.2.4 Radiation Chemistry of Carbohydrates

Radiolysis of water generating free radicals may affect the carbohydrate structure and composition. The major radiolysis effect is caused by OH radical; abstracting a·H atom from a carbohydrate's carbon, thus producing an α-hydroxyl radical. This radical may contribute to other reactions producing at the end stable products (e.g. production of gluconic acid from irradiated glucose) (Phillips & Moody, 1959). Upon irradiation of carbohydrate solutions, the pH may decrease due to the formation of acidic compounds (Dauphin & Saint-Lebe, 1977; Simic, 1983). Irradiation of sugars may involve gas production (e.g. H_2 and CO_2) (Dauphin & Saint-Lebe, 1977) as well as the formation of carbonyls (e.g. formaldehyde, acetaldehyde, and malonaldehydes) (Xuetong, 2012).

7.3 Application of γ-Irradiation

7.3.1 Food Fermentation

Food irradiation represents a promising technique among few others addressing both food safety and quality due to its ability to control spoilage and foodborne pathogens, without significantly affecting the organoleptic properties of the food product (Sadecka, 2010; Verde et al., 2013). Figure 7.1 shows an example of

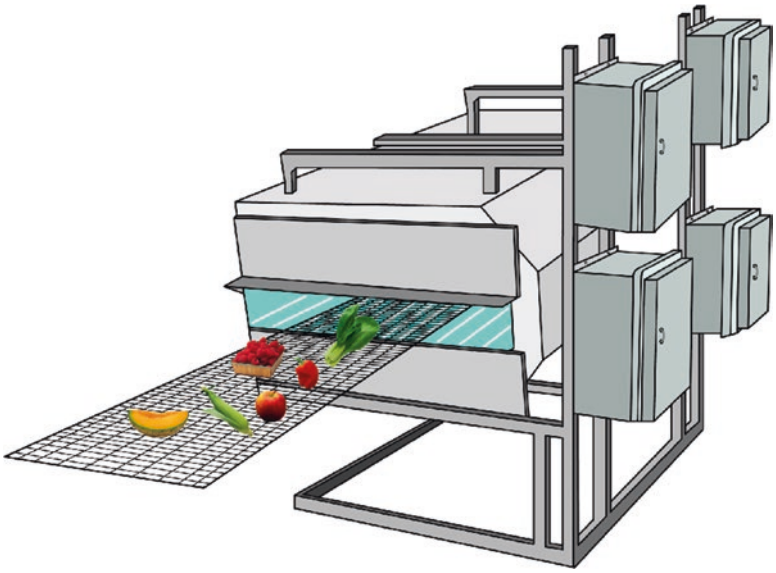

Fig. 7.1 Schematic representation of an example of equipment used for radiosterilization of food products at industrial scale

schematic equipment for γ-irradiation of food products at industrial scale. Studies have demonstrated the effect of γ-rays in preserving the food products. γ-Irradiation of Kimchi, a Korean salted and fermented vegetables, and its impact in extending the shelf-life was evaluated (Song et al., 2004). The authors found a lower lactate dehydrogenase (LDH) activity and delaying acidification when applying up to 10 kGy γ-irradiation at the early stage of Kimchi fermentation with a dose-dependent effect on the inactivation of fermentative microorganisms. When applying γ-irradiation on the mid-fermentation stage of Kimchi, the authors observed effective inactivation of microorganisms, LDH exhibited high activity and the acidification continued. It has been reported through this study that the samples treated at 10 kGy were less appreciated than those treated at either 2.5 or 5 kGy, concluding thus that γ-irradiation at up to 5 kGy on the early fermentation stage can preserve the organoleptic quality of Kimchi.

The shelf-life of minimally processed cabbage and cucumber through γ-irradiation was studied by Khattak et al. (2005). Microbial safety, texture, and sensory quality were investigated for minimally processed, polyethylene-packed, and irradiated cabbage and cucumber. The samples were stored at 5 °C for 2 weeks, with firmness values ranging from 3.23 to 2.82 kg, for the control and the irradiated cucumbers at 3 kGy. Gradual decrease in firmness was observed when increasing the radiation dose (up to 3 kGy). Either after applying 3 kGy irradiation dose or after 14 days storage, cucumbers softened, whereas acceptable texture was obtained when samples were treated with up to 2.5 kGy dose, and no effect on the appearance

scores of cabbage was observed. The authors reported a better overall acceptability of cucumbers at 2.5 and 3 kGy irradiation doses, contrary to the appearance and flavor, which decreased for the higher irradiation doses.

The effects of γ-irradiation on sensory qualities, microbial population, and chemical properties of salted and fermented squid (*Todarodes pacificus*) were investigated (Byun et al., 2000). After salting with 5, 10, and 20 % (w/w) sodium chloride, squid slices were γ-irradiated with 2.5, 5, and 10 kGy doses then fermented at 15 °C for 50 days, and compared to nonirradiated samples. The authors concluded that the sensorial properties, bacterial populations, and pH values were depending to salt concentration and irradiation doses. When combining low salt concentration (10 %) with γ-irradiation, effective squid fermentation and shelf-life extension were observed compared to control without food additives.

In another study performed by Byun, Son, Yook, Jo, and Kim (2002), the physiological activity of Korean soybean fermented foods under γ-rays was studied. The authors compared the γ-irradiation of Chungkookjang and Doenjang; the whole cooked soybean product and the soybean paste, respectively. They applied the γ-irradiations at doses of 5, 10, and 20 kGy and evaluated the physiological activity by analyzing the inhibition of the angiotensin-converting enzyme, xanthine oxidase, and tyrosinase, as well as by testing the free radical scavenging ability. The authors demonstrated that up to 10 kGy, no significant changes on physiological activities were noticed. The irradiation effects on biogenic amines in Korean fermented soybean paste during fermentation was studied by Kim et al. (2003). The authors prepared and irradiated soybean paste at 5, 10, and 15 kGy, followed by fermentation for 12 weeks at 25 °C. Results showed a decrease in *Bacillus spp.* and lactic-acid bacteria after irradiation but their increase during fermentation. Biogenic amine contents between control and irradiated samples were not significant before fermentation. However, some of them were showing significant decrease during fermentation, for irradiated samples. The potential applications of γ-irradiation and fermentation are summarized in Table 7.1.

7.3.2 Alcohol Fermentation

Radiopasteurization method was applied to molasses fermentation media and its effect on fermentation and ethanol production was studied by Iizuka, Shibabe, and Ito (1969). The authors reported a decrease of viability of yeast in molasses to 70 % by heating at 80 °C for 30 min, to 10 % and 1 % by irradiation with 3.0×10^5 rad and 6.0×10^5 rad, respectively. The growth rate and performance of *Saccharomyces cerevisiae* in irradiated mash were similar to those observed by heat-pasteurized mash. In the absence of nitrogen in the molasses mash, 14 % difference in fermentation yield was observed between heating at 80 °C and irradiation with 3×10^5 rad. Furthermore, when nitrogen was supplemented to the molasses mash, the fermentation rate and ethanol production were higher upon irradiation compared to heating. The effect of γ-irradiation on alcohol production from corn was also studied

Table 7.1 Potential applications of γ-irradiation on fermented food products

Food product	Treatment conditions	Major findings	References
Kimchi (korean salted and fermented vegetables)	2.5, 5, and 10 kGy	γ-irradiation at up to 5 kGy on the early fermentation stage provides both stopping fermentation and preserves the organoleptic quality of Kimchi	Song et al. (2004)
Cabbage and cucumber	Irradiation dose up to 3 kGy	A better overall acceptability of cucumbers and cabbage, when applying 2.5 and 3 kGy irradiation doses	Khattak et al. (2005)
Salted and fermented squid (*Todarodes pacificus*)	2.5, 5, and 10 kGy	When combining low salt concentration (10 %) with γ-irradiation, effective squid fermentation, and shelf-life extension were observed compared to control without food additives	Byun et al. (2000)
Korean soybean fermented foods	5, 10, and 20 kGy	Up to 10 kGy, no significant changes on physiological activities were noticed	Byun et al. (2002)
Korean fermented soybean paste	5, 10, and 15 kGy	A decrease in *Bacillus spp.* and lactic-acid bacteria after irradiation followed by an increase during fermentation	Kim et al. (2003)

(Han, Cho, & Ciegler, 1983). Different doses of γ-rays (0–100 Mrad) were applied to cracked corn in order to evaluate their effects on sugar yield, enzymatic hydrolysis of starch, growth of yeast, and production of alcohol. The authors found that beyond 50 Mrad, the amount of reducing sugars increased significantly; however, small amount of glucose was observed. It was found that when γ-irradiation was applied at lower doses, the susceptibility of corn starch to enzymatic hydrolysis increased significantly, giving about 12.5 % reducing sugar after amylase treatment without cooking. This amount was higher than that produced from cooked (gelatinized) corn by the same enzyme treatment. The authors found similar level of alcohol produced on uncooked/irradiated, and cooked corns. They concluded that the conventional cooking process prior to saccharification could be replaced by irradiation which provides additional benefit of sterilization of the medium.

The influence of γ-irradiation on ethanol production from yeast was studied later by Del-Mastro, Gimenes, and Villavicencio (1988). γ-irradiation was applied at up to 6 kGy to investigate the fermentative capacity of two yeast strains. The authors reported unchanged ethanol production using irradiated cells at 3 kGy; however, reduced yield by 43 % was observed for one strain treated at 6 kGy. They suggested the radio-resistant process of yeast to convert sugar to alcohol, and the most important conclusion is that these cells are not affected by γ-rays when treating the fermentation medium. Similarly, the fermentation of irradiated "sugarcane must" was studied by Alcarde, Walder, and Horii (2003). The authors investigated the influ-

ence of γ-irradiation in reducing the bacterial population (*Bacillus* and *Lactobacillus*) usually contaminating the ethanolic fermentation by yeasts. Lethal radiation dose benefits on some parameters of fermentation were also verified. Contaminated sugarcane must with *Bacillus* and *Lactobacillus* bacteria was irradiated with 2, 4, 6, 8, and 10 kGy γ-rays. Total acidity produced during ethanolic fermentation by *Saccharomyces cerevisiae* was evaluated, as well as yeast viability and ethanol yield. The authors reported reduced bacterial population upon irradiation and decreased acidity as the dose rate of radiation increased.

7.4 New Perspectives

Apart from the food preservation, γ-irradiation can be used in several processes related to fermentation. Some of the most important applications are summarized in Fig. 7.2. For instance, the ability of γ-irradiation to reduce the cost of producing fermentation-based drugs has been recently described. Moreover, γ-irradiation can be used to improve alcohol fermentation, thus eliminating steps in the process (i.e. elimination of heat gelatinization) (Han, 1983). In addition, γ-irradiation has been widely applied for the generation of genetically modified fermentative strains to enhance the production of ethanol through fermentation, among many other applications. In fact, although medium irradiation by γ-rays leads to its sterilization, by killing bacterial contaminants, the impact on yeast remains lower than that noticed for bacteria. γ-Irradiation of yeast was primarily used to generate highly resistant

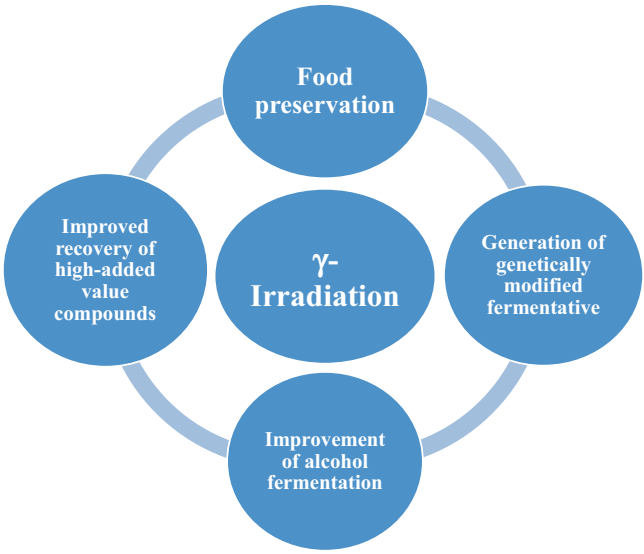

Fig. 7.2 Current and potential applications of γ-irradiation in fermentation-related processes

strains to temperature and ethanol concentration, which are more suitable for industrial applications. For instance, *Saccharomyces cerevisiae* strains were screened after γ-irradiation for their tolerance to temperature and ethanol during fermentation (Mehdikhani, Rezazadeh Bari, & Hovsepyan, 2011). The authors irradiated with γ-rays at different doses (0.1, 1, 2, 3, 4, 5, and 10 kGy/h) three yeast strains and screened them to grow and ferment molasses in a range of 35–45 °C. Two strains were able to grow at 42 °C, and their efficiency for ethanol tolerance was significantly higher compared to control.

In a similar study, 25 strains of *Saccharomyces cerevisiae* highly tolerant for sugar and ethanol as well as resistant to inhibitors were screened (Jang, Lim, & Kim, 2014). The authors reported that the strain *S. cerevisiae* KL5-G2 was selected after γ-irradiation and was showing improved features for inhibitor (mixture of 75 mM formic acid, 75 mM acetic acid, 30 mM furfural, 30 mM hydroxymethyl furfural, and 2.7 mM vanillin), and high concentration of ethanol resistances. The authors selected this strain after sequential transfer of survival strains on increasing inhibitor cocktail concentrations, showing growth in YNB medium with up to 80% inhibitor cocktail, whereas the parental KL5 strain could not grow at all. After γ-irradiation and sequential adaptation, one strain; KL5-G2-A9 was selected to produce the highest ethanol yield in a complex YP medium containing 60% inhibitor cocktail and 5% glucose, with 0.304 g/L/h, compared to only 0.072 g/L/h for the non-irradiated strain KL5.

Conclusions

Several studies have demonstrated the potential of γ-irradiation to preserve fermented products, thus maintaining nutritional and physicochemical properties. Minor changes are occurring during treatment with γ-irradiation, mainly produced by the generation of free radicals, but remain lower than that occurring naturally. γ-Irradiation technology has been extended for a wide range of applications, including the enhancement of fermentation features by either treating the medium or genetically modifying strains to ameliorate the fermentative characteristics. Although the potential use of γ-irradiation for food product preservation and the economical benefits drawn, its application remains limited, and more consumer awareness should be deployed.

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Chapter 8
Novel Thermal Technologies and Fermentation

Mohamed Koubaa, Elena Roselló-Soto, Sonia Barba-Orellana, and Francisco J. Barba

8.1 Introduction

Traditionally, thermal treatment has been used to reduce the microbial load and inactivate deleterious enzymes since many of the quality changes that occur during distribution and storage of these foods are due to reactions catalyzed by enzymes, such as peroxidase (POD), polyphenol oxidase (PPO), and pectin methylesterase (PME). However, thermal processing can also alter sensorial (texture, taste, flavor, and color) and nutritional quality attributes, such as reduction of some bioactive compounds (Mañas & Pagán, 2005). For these reasons, it is desirable to keep thermal treatment conditions at a least possible level sufficient for inactivation of the deleterious enzymes, in order to minimize the quality losses. Novel electromagnetic technologies represent the potential to fully or partially replace the conventional heating processes, and gained more and more interest for industrial applications (Vicente & Castro, 2007). Such novel thermal technologies are considered as volumetric form of heating. In fact, thermal energy is directly generated inside the foods, and no limitations are associated to heat transfer coefficients. Besides these technological features and the reduction of processing

M. Koubaa
Sorbonne Universités, Université de Technologie de Compiègne, Laboratoire Transformations Intégrée de la Matière Renouvelable (UTC/ESCOM, EA 4297 TIMR), Centre de Recherche de Royallieu, CS 60319, 60203 Compiègne Cedex, France

E. Roselló-Soto • F.J. Barba (✉)
Faculty of Pharmacy, Nutrition and Food Science Area, Universitat de València, Avda. Vicent Andrés Estellés, s/n 46100 Burjassot, València, Spain
e-mail: francisco.barba@uv.es

S. Barba-Orellana
Centro Sanitario Integrado de Xirivella, Consorci Hospital General Universitari València, C/ Virgen de Los Desamparados, s/n 46950, Xirivella, Valencia, Spain

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time, the application of these novel thermal technologies preserves the nutritional, sensorial, and organoleptic properties of processed foods (Cullen, Tiwari, & Valdramidis, 2012). At this stage of development, many technological developments have been directed towards unit operations such as pasteurization, sterilization, cooking, and drying, and currently the new technological approaches for food preservation are serious candidates to replace the traditional well-established preservation processes.

Fermentation is one of the oldest methods practiced by human being for the transformation of some food products in order to extend their storage period or shelf-life and give them novel organoleptic properties. As an example of these products, yoghurt is one of the widely transformed dairy foods by lactic acid bacteria. During the storage period, the post-acidification process is a major problem, as it changes the properties of the final product (Rajapaksha, Kodithuwakku, Silva, & Rupasinghe, 2013). Inactivating, even partially the microbial load after fermentation is of great importance to preserve the final food quality. However, when applying traditional thermal processes, changes that are primarily related to the transformation of thermolabile compounds to other products may occur. In this line, novel thermal technologies (e.g., radio frequency, microwave heating, and ohmic heating) have appeared as a potential tool to replace conventional thermal treatments in fermented food products. Their action, although leads to the increase of temperature inside the treated products, could be associated with other phenomena (i.e., cell membrane electroporation using ohmic heating), which accelerate the microbial load inactivation within the fermented product. This chapter deals with the application of three novel thermal technologies: radio frequency (RF), microwave heating, and ohmic heating, in food fermentation processes.

8.2 Radio Frequency and Fermentation

Alternating electric field created between the two electrodes of RF generator causes the continuous reorientation of water molecules in the conveyed materials being treated (in bags or in bulk (Fig. 8.1)) to face opposite poles. The phenomenon looks like the behavior of magnetic bars in an alternating magnetic field. The movement of molecules within the treated product and the friction between them causes the rapid heat of the material throughout its entire mass.

The application of RF heating in food industry has become an interesting technique and increasingly taking advantages compared to microwave heating (Pereira & Vicente, 2010; Piyasena, Dussault, Koutchma, Ramaswamy, & Awuah, 2003; Ramaswamy & Tang, 2008). The dielectric heating process between both technologies, RF (3 kHz–300 MHz) and microwave (300 MHz–300 GHz), is similar and based on the induction of molecular vibrations within the product (Ramaswamy & Tang, 2008). Nevertheless, in terms of uniformity and energy consumption, RF treatment provides more uniform heating and consumes less energy than MW (Piyasena et al., 2003). The dielectric properties of microbial suspensions related to the application of RF are proportional to the particles' radius and volume fraction constituting the suspended phase (Harris et al., 1987).

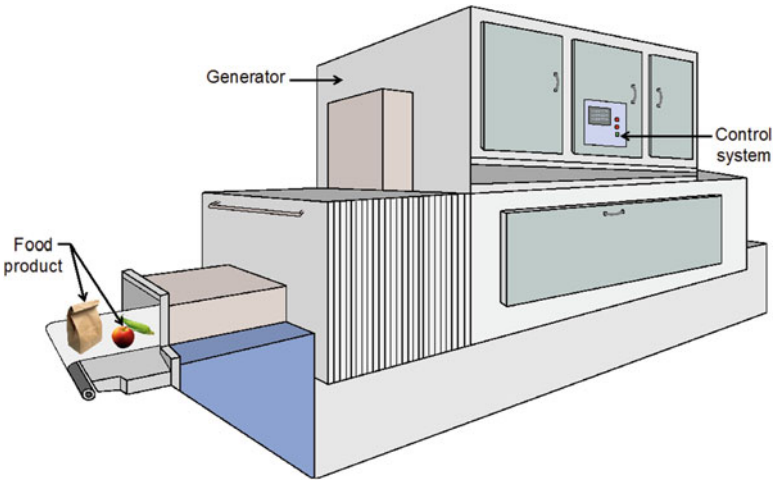

Fig. 8.1 Schematic representation of an example of radio frequency equipment used at industrial scale

RF heating is not only applied for bulk products but also developed to treat packed food materials. Such equipment are available to treat packed products (e.g. pea starch, proteins, flax meal, nuts, spices) including prepared foods. The effect of RF treatment on yoghurt after the fermentation process has been evaluated and compared to conventional heating (Siefarth et al., 2014). The authors concluded that both heating processes gave homogenous distribution of the temperature and are efficient to avoid the development of contaminants in the final product. However, RF method was more interesting compared to conventional treatment due to its faster heating (0.3 K s^{-1}). In addition to energy consumption aspect, the study was also focused on yoghurt shelf-life, pH, color, and sensorial quality. Due to the mildness behavior of RF, lactic acid bacteria (LAB) were partially present after the heating treatment. Though it is present, it was reported that the shelf-life of yoghurt was extended due to the reduction of LAB content and the complete inactivation of fungi. The reduction of LAB content was associated with the prevention of the post-acidification process. The authors reported no changes in the pH values and sensorial quality properties of the RF treated yoghurt, but somewhat slight color changes probably related to the caramelization processes due to heating. The study demonstrated its potential for post-heating of fermented yoghurt.

The dielectric permittivity of microbial suspensions at radio frequencies has been reported many years ago as a novel method for the real-time estimation of microbial biomass (Harris et al., 1987). The described method demonstrates the possibility to follow in situ and in real time the microbial growth during the fermentation process. The originality of the approach to estimate the biomass is related to its noninterference with noncellular particulate matter, and its linearity at up to two-fold higher volume than that obtained by Beer–Lambert law.

8.3 Ohmic Heating and Fermentation

Ohmic heating (OH) is the process employed to heat materials when electric current is passed through them (Fig. 8.2). In OH, the direct dissipation of energy into the food product avoids the solid-liquid contact to transfer the heat or inside solid particles (Knirsch, dos Santos, de Oliveira Soares Vicente, & Vessoni Penna, 2010). OH has been reported for various food processing applications including blanching, evaporation, dehydration, fermentation, extraction, sterilization, and pasteurization (Sastry et al., 2009). One of the main advantages of OH is that it is an environmental-friendly technology which allows preserving better sensorial and nutritional properties compared to conventional treatments. Moreover, some of the other benefits of this technology are derived from its continuous operability, without heat transfer surfaces; therefore, a system can easily be implemented into existing processing lines. In addition, during OH processing, there is a reduced over heating effect or surface fouling of the product compared to conventional heating and energy lost during heating food products is minimized (no residual heat transfer after the current is turned off). Moreover, this technology is a useful tool in preheating products before canning. On the other hand, when OH is used in food preservation (e.g. HTST), it is possible to obtain the desired temperature very quickly. This technology presents a low maintenance costs (no moving parts) and high energy conversion efficiencies (Pereira & Vicente, 2010).

It has been reported in literature that the application of electric field under OH may involve the electroporation of cell membranes; which limits some commercial exploitation of specific products (Sastry, 2005). However, this feature is of great interest especially when the electroporation of cell membrane is targeted. In fact,

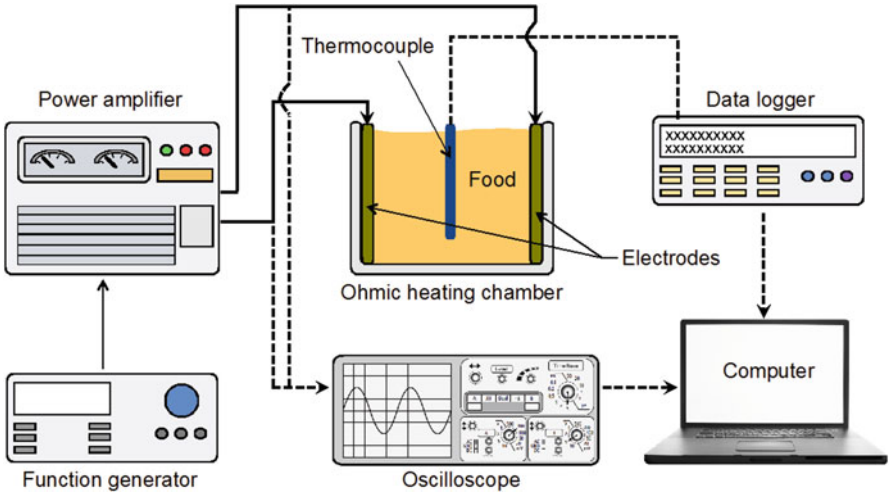

Fig. 8.2 Schematic representation of Ohmic heating equipment for food applications

the electroporation effect caused by OH after applying sublethal temperatures has showing promising results for fermentative processes using *Lactobacillus acidophilus* (Cho, Yousef, & Sastry, 1996; Loghavi, Sastry, & Yousef, 2008, 2009). However, despite the potential benefits of this technology, there is a lack of information about its application in fermentation-related processes. One of these studies was performed by Cho et al. (1996) who compared the fermentative process using conventional heating by water bath, and OH at a constant voltage value of either 15 or 40 V. The authors conducted the processes at different temperatures (30–40 °C) and observed a reduction of the lag fermentative phase as an incidence of cell membrane electroporation through electric field application. This behavior was explained by the efficiency of nutrient transportation inside the cells.

8.4 Microwave Heating and Fermentation

Microwave heating is a technology based on electromagnetic radiations with a frequency from 0.3 to 300 GHz. Several potential applications of microwaves related to fermentation processes have been explored by various authors in the available literature. Among them, microwave-assisted extraction (MAE) is a promising application (Chemat, Vian, & Cravotto, 2012; Rombaut, Tixier, Bily, & Chemat, 2014), particularly as pre-treatment in the extraction of high added-value compounds such as lipids (Dai, Chen, & Chen, 2014), pectins (Prakash Maran, Sivakumar, Thirugnanasambandham, & Sridhar, 2014), anthraquinones (Hemwimon, Pavasant, & Shotipruk, 2007), essential oils (Farhat, Fabiano-Tixier, Visinoni, Romdhane, & Chemat, 2010), and polyphenols (Aspe & Fernandez, 2011). Moreover, other authors have also explored the feasibility of microwaves to be used in other fermentation-related processes (e.g. production of hydrolytic enzymes, biohydrogen production, shorten fermentation times, reduced cumulative gas production, preservation of fermented products) (Fig. 8.3). MAE is based on direct effect on molecules by ionic conduction and dipole rotation (Pérez, Conde, & Domínguez, 2014). When MAE is applied, the absorption of energy in the sample and especially by polar molecules such as water (moisture content) leads to cell disruption, which can facilitate the fermentation processes. A schematic representation of a microwave laboratory system is illustrated in Fig. 8.4. However, at this stage of development there is a lack of information about the commercial feasibility of these processes. Some of the main findings concerning the potential applications of microwaves and related to fermentation processes are listed below.

Limited studies have shown the effect of microwaves to improve fermentation-related processes. For instance, the effects of different treatments (conductive, microwave, yeast, conductive + yeast, microwave + yeast) during the fermentation process of grape were investigated in order to evaluate if the fermentation time can be reduced (Kapcsandi, Nemenyi, & Lakatos, 2013). For this purpose, the fermentation period was studied in samples with respect to changes in sugar, alcohol, and

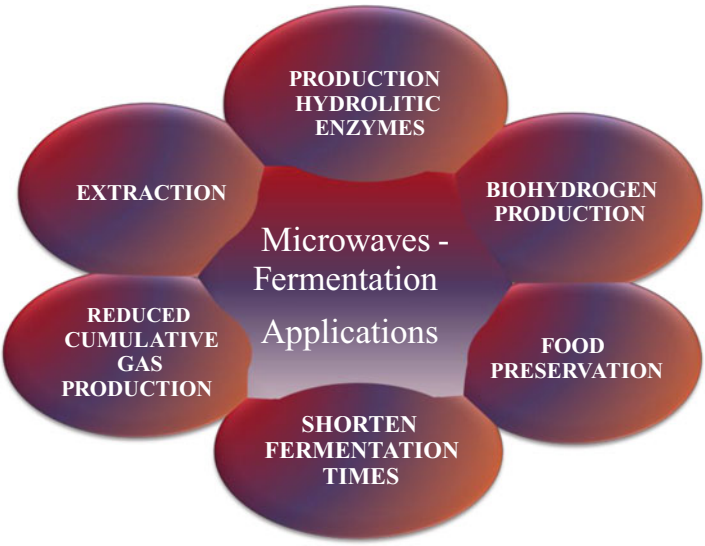

Fig. 8.3 Potential applications of microwaves related to fermentation

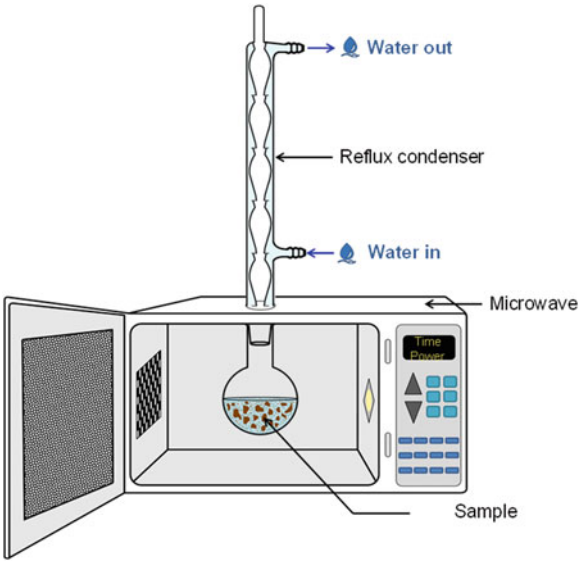

Fig. 8.4 Schematic representation of microwave equipment for food applications used at laboratory scale

acidity. The authors found that the fermentation time was reduced up to 40 % when the samples were pretreated with microwaves at the optimum conditions compared to the control samples.

In another study, the effects of microwave maceration with early pressing and co-inoculation of yeast and malolactic bacteria for simultaneous alcoholic and malolactic fermentation was evaluated (Carew, Close, & Dambergs, 2015). For this purpose, the authors co-inoculated the yeasts *Saccharomyces cerevisiae* RC212 and

EC1118, and *Saccharomyces bayanus* AWRI1176 with *Oenococcus oeni* PN4, immediately after must microwave maceration. They found that alcoholic and malolactic fermentation were completed after 17 days postinoculation for all three yeast treatments. Moreover, they also found that the AWRI1176-treated wines had approximately twice the non-bleachable pigment and color density of wines fermented by EC1118 and RC212 at 16-month bottle age. The authors concluded that the application of microwave treatment can shorten the fermentation time.

On the other hand, the impact of microwave irradiation (1000 W, 0–5 min) on the fermentation characteristics and nutritive value of tomato pomace for ruminants using in vitro gas production technique was studied (Maheri-Sis, Eghbali-Vaighan, Mirzaaghazadeh, & Ebrahimnezhad, 2012). The authors concluded that microwave irradiation reduced cumulative gas production volume during fermentation at early incubation times.

Other authors evaluated the impact of microwave pre-treatment on enzyme production during solid-state fermentation of wheat by *Aspergillus oryzae* (Melikoglu, 2012). The industrial production of hydrolytic enzymes is of paramount importance in order to operate successful cereal-based biorefineries, where macromolecules in cereals (such as starch, proteins, and phosphorus) are converted into their monomers (glucose, free amino acids, and free phosphorous, respectively) for the production of different value-added products (Melikoglu, 2012). For instance, the authors found that microwaves may be a useful tool to improve the production of hydrolytic enzymes as they found a significant increase in glucoamylase and protease activities after microwave pre-treatment (30 s) compared to untreated samples.

In addition, the use of microwaves as pre-treatment for improving fermentation processes, thus facilitating the production of valuable compounds (e.g. biohydrogen production) represents a huge potential in terms of renewable energy exploitation (Saleem, Omar, Kamal, & Biak, 2015). For instance, the effects of microwave-assisted pre-treatment of lignocellulosic biomass immersed in alkaline glycerol for fermentable sugar production was investigated (Diaz et al., 2015). These authors applied a microwave pre-treatment to enhance enzyme hydrolysis of corn straw and rice husk immersed in water, aqueous glycerol, or alkaline glycerol. They found a significant improvement in saccharification yields, for both corn straw and rice husk, when biomass was microwave pre-treated in alkaline glycerol compared to commercial cellulose cocktail Celluclast®. In addition, they also found significant changes in the structure of corn straw subjected to microwave pre-treatment.

8.5 Conclusions

Novel thermal technologies have the potential to be used in a wide range of fermentation-related applications, including the enhancement of fermentation features as a pre-treatment or to improve the shelf-life of fermented food products. For instance,

radio frequency was shown as a promising tool to reduce and to achieve the complete inactivation of fungi from yoghurt, thus improving its shelf-life along with improved sensorial and nutritional properties compared to conventional thermal treatments. In addition, the potential of microwave treatment to shorten fermentation process during winemaking was demonstrated. Moreover, this technology may be an important tool to be used as pre-treatment in fermentation processes, thus facilitating the production of valuable compounds. Although the potential use of novel thermal technologies seems to be evident in fermentation processes, there is a lack of information and their applications remain limited. Therefore, and further studies dealing with this topic are needed.

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Chapter 9
Novel Fermented Dairy Products

Spasenija D. Milanović, Dajana V. Hrnjez, Mirela D. Iličić, Katarina G. Kanurić, and Vladimir R. Vukić

9.1 Introduction

Consumption of fermented milk products, as a healthy and nutritious food has a long tradition. Initially, the function of milk fermentation was to extend the shelf-life of the product and additionally many benefits are achieved, such as improving the digestibility and flavour, as well as the ability to produce a wide range of different products. Historically, fermentation process involved unpredictable and slow souring of milk caused by the indigenous microflora in the milk. However, applications of modern microbiological processes have resulted in the production of various fermented dairy products under controlled conditions.

Currently, around 400 different names are applied for traditionally and industrially produced fermented milk products. The specificity of each type of product is reflected by the applied type of microorganisms, milk and process conditions (Surono & Hosono, 2011). The growing consumer's interest in fermented dairy products is gaining due to the development of new food processing techniques, scientific evidence of its health benefits as well as changing social attitudes (Korhonen & Pihlanto, 2006). Novel trends in the market of fermented dairy products are leading to obtain various products with high functional and nutritive but less energy value, as well as products more suitable for special allergenic nutrition. Therefore, various functional ingredients, such as probiotics and prebiotics, are applied in certain manufacture (Matilla Sandholm et al., 2002; Tamime, 2006).

S.D. Milanović • D.V. Hrnjez (✉) • M.D. Iličić • K.G. Kanurić • V.R. Vukić
Faculty of Technology, University of Novi Sad, Novi Sad, Serbia
e-mail: dajana@tf.uns.ac.rs

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Recent efforts in the development of new fermented dairy products could be divided into two groups in an aim to:

1. Obtain the products with improved physicochemical characteristics, rheology, texture, microstructure and sensory properties,
2. Get the products with pronounced nutritive characteristics and functionality.

Some of these specific desired characteristics, such as manufacture of low-fat or non-fat (fat-free) products, generally lead to a lack of good physical, rheological, textural and overall sensory properties of the final products. The application of different starter cultures (conventional, non-conventional, probiotics), ingredients (milk proteins, whey protein concentrate, whey protein isolates, enzyme transglutaminase, prebiotics) or some innovative techniques (high-pressure processing, high-pressure homogenization, ultrasonic processing and pulse electric field) could improve functionality and quality of product.

9.2 Diversity of Fermented Dairy Products

Fermentation of milk is one of the oldest methods for food preservation dated from some 10–15,000 years BC. It is likely that the origin of yoghurt was the Middle East (Tamime, 2006). Nowadays, there are numerous types of fermented milk in different parts of the world. On the basis of the starter culture applied, fermented dairy products could be classified into the following types:

1. Products of lactic fermentation where strains of mesophilic or thermophilic lactic acid bacteria (LAB) are used (yoghurt, zabadi, labneh, acidophilus milk, biogarde, bifighurt, ABT, cultured buttermilk, ymer, dahi, filmjölk, tätmjölk, yakult, etc.);
2. Products obtained via alcohol-lactic fermentation (kefir, kumys, acidophilus-yeast milk, skyr);
3. Products where molds grow in addition to microorganisms from fermentation of type 1 and 2 is present (villi, skyr) (Surono & Hosono, 2011; Tamime, 2006; Tamime & Robinson, 2004).

Some established fermented dairy products, types of applied starter cultures, their characteristics and origin are presented in Table 9.1.

The commercial process of yoghurt making uses a defined mixture of lactic acid bacteria, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, but other products may require a different blend of microorganisms. The enzyme of the starter microflora added to milk acts on the native proteins to produce substrates that can be used for metabolism and growth by other starter microorganisms. Metabolites produced during the fermentation cause changes in the pH, texture and taste of the fermented dairy products and may affect the structure of the product. Technological parameters, milk composition and characteristics, synergistic interactions between microorganisms, exopolysaccharides and food ingredients (dietary

Table 9.1 Types of fermented dairy products

Product (origin/ country)	Starter culture	Fermentation *T* (°C)/*t* (h)	Characteristics	References
Acidophilus milk (Great Britain)	*Lactobacillus acidophilus*	38 °C/8–10 h	Liquid consistency, 0.5–2 % milk fat, 1.5–2 % lactic acid	Surono and Hosono (2011), Tamime (1995)
AB Cultura (Denmark)	*Lb. acidophilus* *Bifidobacterium bifidum*	37–40 °C/~10 h	Liquid consistency, 0.4–0.9 % lactic acid	Surono and Hosono (2011)
Ayran (Asia, Middle East, Turkey, Lebanon)	*Streptococcus thermophilus* *Lactobacillus bulgaricus*	40–45 °C/4–5 h	Drinking or stirred yoghurt, low viscosity, yoghurt is diluted with water or whey from labneh (30–50 %), salt is added up to 1 %, stabilizers, pH ~ 4.6, 9.5 % total solids, 1.5 % milk fat, 0.4 % titratable acidity	Erkaya, Baslar, Sengül, and Ertugay (2015), Nilsson, Lyck, and Tamime (2006)
Biogarde^{R} (Germany)	*Sc. thermophilus*, *Lb. acidophilus*, *Bif. bifidum*	37–40 °C/10–12 h	Set product, high consistency, slightly sour taste, 0.4–0.9 % lactic acid	Surono and Hosono (2011), Tamime (1995)
Bulgarian Buttermilk (Bulgaria)	*Lb. delbrueckii* subsp. *bulgaricus*	38–42 °C/10–12 h	Sharp acidic flavour, 1.4 % titratable acidity	Surono and Hosono (2011)
Cultured Buttermilk (Denmark, Netherlands)	Commercial butter starter or a mixture *of*: *Lactococcus lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, citrate-positive, *Lc. lactis* and *Leuconostoc mesenteroides* subsp. *cremoris*	19–22 °C/16–22 h	Mild acid flavour, a smooth viscous body and texture, pH ~ 4.5–4.7; 0.9 % titratable acidity	Fondén, Leporanta, and Svensson (2006), Libudzisz and Stepaniak (2011), Surono and Hosono (2011)

(continued)

Table 9.1 (continued)

Product (origin/ country)	Starter culture	Fermentation *T* (°C)/*t* (h)	Characteristics	References
Dahi/Dadih (India/ Indonesia)	*Sc. thermophilus, Lb. delbrueckii* subsp. *bulgaricus* or *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, citrate-positive *Lc. lactis Lb. casei* subsp. *casei*, *Ln. paramesenteroides*, *Lb. plantarum*, *Enterococcus faecium* (depends on the place of manufacture)	22–25 °C/16–18 h, 28–30 °C/24 h	Semisolid product made from cow's milk, bovine or buffaloes milk, mild acid, 18.5 % total solids, 0.9 % titratable acidity	Akuzawa, Miura, and Surono (2011), Surono and Hosono (2011), Yadav, Jain, and Sinha (2007)
Filmjölk (Nordic/ Scandinavian fermented milks/ Sweden, Norway, Denmark)	*Lc. lactis* subsp. *lactis* citrate-positive *Lc. lactis*, *Ln. mesenteroides* subsp. *cremoris*	17–24 °C/17–24 h	Semisolid milk, mild slightly acidic, characteristic aroma, high viscosity, pH ~ 4.3–4.4, 0.1–4.5 % milk fat	Fondén et al. (2006), Surono and Hosono (2011), Roginski (2011)
Kishk (Egypt, Middle Eastern Country)	*Streptococcus lactis*, *Streptococcus cremoris*	37 °C/24 h	Dried fermented milk products with or without cereals and other additives (vegetables, spices, herbs or fruits), 3–16 % moisture, 8.9–54.5 % protein, 1.6–19.9 % fat, 31–65 % carbohydrate, 0.5–2.5 dietary fibre, 1–2.5 % titratable acidity	Abd El-Salam (2011), Jandal, (1996)
Labneh/Labaneh (Syria/Lebanon)	*Lc. lactis* spp. *lactis*, *Lc. lactis* spp. *cremoris*	43 °C /12–24 h	Concentrated fermented milk smooth spreadable, 22–26 % total solids, 9–11 % milk fat, 8.5–9.0 % protein, 3.5–4 % lactose, 1.5–2.5 % titratable acidity, about 1 % sodium chloride.	Abd El-Salam (2011), Ozer (2006), Surono and Hosono (2011), Tamime, Hasan, Farnworth, and Toba (2007)

(continued)

Tätmjölk (Nordic/ Scandinavian fermented milks/ Sweden, Norway, Denmark)	As for *Filmjölk*, *Lc. lactis* subsp. *lactis* citrate-positive *Lc. lactis*, *Ln. mesenteroides* subsp. *cremoris*	17–20 °C/17–24 h	High viscosity, ropiness, a very mild acid taste and low syneresis, pH ~ 4.3–4.4	Fondén et al. (2006), Surono and Hosono (2011)
Zabadi (Egypt, several Arabic country)	*Sc.thermophilus*, *Lb. delbruecki* spp. *bulgaricus*	37–45 °C/24 h	Plain set type yoghurt made from buffalo milk, 12–18 % total solids, 2.91–4.96 % protein, 2.92–3.98 % lactose, 2.6–3.67 % milk fat, 0.93–1.2 % titratable acidity and 1.67–5.9 mg/kg acetaldehyde	Abd El-Salam (2011), Surono and Hosono (2011)
Yakult (Japan)	*Lb. paracasei* subsp. *paracasei*, Shirota strain	30–37 °C/16–18 h	Liquid-type fermented milks, pH ~3.8–3.9, 82.6 % moisture, 1.2 % protein, 0.15 % milk fat, 15.8 % carbohydrate (14 % added sugar)	Akuzawa et al. (2011), Surono and Hosono (2011), Tamime et al. (2007)
Yoghurt (produced around the world)	*Sc. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus*	42–45 °C/3–6 h	Gel-type fermented milks (set or stirred, drinking with or without addition of non-dairy ingredients such as sweeteners, fruits, vegetables and other naturals flavourings), pH ~ 4.4–4.6, 0.9–1.2 % lactic acid	Robinson and Itsaranuwar (2006), Surono and Hosono (2011), Tamime et al. (2007)
Ymer (Denmark)	*Lc. lactis* spp. *lactis*, *Lc. lactis* spp. *cremoris*, *Lc.mesenteroides* spp. *cremoris*	20–27 °C/16–20 h	Soft milk pudding, a smooth and silky texture, pH ~ 4.5, 11 % non-fat solids, 6 % protein, 3.5 % milk fat	Fondén et al. (2006), Surono and Hosono (2011), Tamime et al. (2007)

(continued)

Table 9.1 (continued)

Product (origin/ country)	Starter culture	Fermentation *T* (°C)/*t* (h)	Characteristics	References
Kefir/German kefir (traditional fermented milk of Asia/Germany)	*Lb. brevis*, *Lb kefir*, *Lb. acidophilus*, *Ln. mesenteroides*, *Ln. cremoris*, *Sc. thermophilus*, *Torulopsis kefir*, *Torulopsis holmi*, *Saccharomyces cerevisiae*, *Candida pseudotropicalis*/ *Lc. lactis* subsp. *lactis*, citrate-positive, *Lc. lactis* subsp. *cremoris*, *Sc. thermophilus*, *Lb. acidophilus*, *Lb. lactis*, *Lb. brevis*, *Candida kefyr*	20–25 °C/24 h, 15–22 °C/24–36 h	Traditional fermented milk of Asia from sheep, goats and buffalo. Commercial kefir is made from cow's milk. A thick drink, a sparkling mouthfeel due to the release in the mouth bubbles of carbon dioxide, 0.9–1.1 % lactic acid, 0.3 % ethanol (0.3–1.0 % alcohol), 1 % carbon dioxide	Rattray and O'Connell (2011), Surono and Hosono (2011), Tamime et al. (2007), Wszolek, Kupiec-Teahan, Skov-Guldager, and Tamime (2006)
Koumiss/Kumys, Kumiss/Kumys (traditional fermented milk of Asia), Europe	*Lb. delbrueckii* subsp. *bulgaricus*, *Lb. kefiranofaciens*, *Lb. lactis*, *Lb. acidophilus*, *Lb. lactis* spp. *lactis*, *Saccharomyces lactis*, *Kluyveromyces lactis*/*Saccharomyces lactis*, *Torula koumiss*	20–25 °C/12–24 h, 30–37 °C/7–15 h	Traditionally produced by fermentation of mare's milk, made from blending cow's skimmed milk and cheese whey. Slightly sparkling mouthfeel, a clean, refreshing and slightly yeasty taste, 0.6–1 % lactic acid, 0.7–2.5 % alcohol (depending on the duration of fermentation)	Surono and Hosono (2011), Tamime et al. (2007), Unicake-Lowe, (2011), Wszolek et al. (2006)
Skyr (Iceland)	*Sc. thermophilus*, *Lb. delbruecki* spp. *bulgaricus Lb. helveticus*, *Saccharomyces*, *Torulopsis*, *Candida*, spp.	39–43 °C/4–6 h	Concentrated fermented milk, soft texture and creamy taste, pH ~ 3.8–4.0, 18–20 % totals solids	Fondén et al. (2006), Tamime et al. (2007)
Viili (Finland, originate in Sweden)	*Lc. lactis* subsp. *cremoris*, *Lc. lactis* subsp.*lactis* biovar *diacetylactis*, *Ln. mesenteroides* subsp. *cremoris*, plus *Geotrichum candidum*	18–21 °C/20 h	Viscous product, mild flavour, with a stronger creamier flavour note at the surface, 2.5–3.9 % milk fat, 0.9 % lactic acid	Fondén et al. (2006), Surono and Hosono (2011), Tamime et al. (2007)

T temperature, *t* time

fibre-inulin, oligofructose, milk proteins, pectin, starch, transglutaminase and others) have been reported to influence the structural and nutritional characteristics of fermented dairy products (Bönisch, Huss, Lauber, & Kulozik, 2007; Bönisch, Huss, Weitl, & Kulozik, 2007; Bönisch, Lauber, & Kulozik, 2007; Crispín-Isidro, Lobato-Calleros, Espinosa-Andrews, & Vernon-Carter, 2014; Debon, Prudencio, & Petrus, 2010; Gauche, Tomazi, Barreto, & Bordignon-Luiz, 2009; Girard & Schaffer-Lequart, 2007; Guyot & Kulozik, 2011; Iličić, Milanović, Carić, Dokić, & Kanurić, 2014; Krzeminski, Prell, Busch-Stockfisch, Weiss, & Hinrichs, 2014; Marafon, Sumi, Alcântara, & Oliveira, 2011; Mende, Peter, Bartels, & Jaros, 2013; Meyer, Bayarri, Tárrega, & Costell, 2011; Milanović, Carić, Đurić, Iličić, & Duraković, 2007; Milanović, Iličić, Djurić, Dokić, & Kanurić, 2009; Prasanna, Grandison, & Charalampopulous, 2013; Ramirez-Santiago, Ramos Solis, Lobato-Calleros, & Alvarez-Ramirez, 2010; Souza Oliveira, Perego, Nogueira de Oliveira, & Converti, 2011; Stijepić, Milanović, Djurdjević-Milošević, & Vukic, 2012).

9.3 Nutritive and Structural Characteristics

9.3.1 Probiotics

In addition to yoghurt culture, probiotic application of starter cultures in fermented dairy products manufacture is becoming more and more interesting because of their therapeutic and prophylactic characteristics. These bacteria beneficially affect human health by improving the balance of intestinal microflora. The two most important genera in the probiotic field are *Lactobacillus* and *Bifidobacterium*, but some others contain species of interest, e.g. *Pediococcus*, *Enterococcus* and *Lactococcus*. For selection, preferably the microbes should have GRAS (Generally Regarded As Safe) status, a long history of safe use in foods, be non-pathogenic, and acid and bile tolerant (Doder, Vukić, Hrnjez et al., 2013; Morgensen et al., 2002).

Parameters that affect the growth of probiotic bacteria in yoghurt production are presented in Table 9.2 (Charalampopoulos & Rastall, 2009).

To successfully develop fermented dairy products containing probiotics, it is important to understand the growth characteristics of probiotics in order to optimize their survival during processing conditions. One of the major limitations of the incorporation of *Bifidobacteria* is the pH value of product and the aerobic conditions of production (Boylston, Vinderola, Ghoddusi, & Reinheimer, 2004; Matilla Sandholm et al., 2002; Milanovic, Panić, & Carić, 2004). In practical application, most strains of *Bifidobacteria* are sensitive to pH values below 4.6. Because of that, the pH value in the final product must be maintained above 4.6. Therefore, fermented milks are not optimal for the maintenance of high concentration of some strains. Certain approved strains of *Lb. acidophilus* and bifidobacteria are the most widely used bacteria during the manufacture of probiotic yoghurts. It is well documented that probiotic bacteria grow slowly in milk because they are devoid of proteolytic enzymes and

Table 9.2 Parameters which affect the growth of probiotic bacteria in yoghurt production (Charalampopoulos & Rastall, 2009)

Milk bland	Fermentation	Storage
• Animal source	• Compatible starter	• pH (plain yoghurt and after fruit addition)
• Pre-processing storage time of raw milk	• Form of starter or probiotic (liquid, DVI)	• Moment of inoculation of probiotic
• Non-fat solids	• If dried DVI, rehydration parameters (solids, temperature, time)	• *L. bulgaricus* content and activity
• Fat content	• Inoculation level of starter or probiotic (CFU/mL)	• Redox level. Additional of antioxidants
• Growth supplements	• Moment of inoculation of probiotic	• Packaging, particularly with respect to oxygen permeability
• Sugar level	• Fermentation temperature	• Encapsulation
• Flavours and fruits	• Fermentation time	
• Preservatives		
• Heating parameters		
• Redox level		

contribute poor sensory and rheological characteristics of product; hence, the practice is to blend these organisms with yoghurt starter (Marafon et al., 2011).

The influence of different combinations of cultures, *Lb. acidophilus* and *Bifidobacterium animalis* subsp. *lactis* as pure cultures or in co-culture with *Sc. thermophilus*, on fermented milk during fermentation, including changes to the acidification profile, organic acid production and lactose consumption and during 28-day storage at 4 °C, in terms of bacteria viability, syneresis, sensory properties, organic acid content and viability under simulated in vitro gastrointestinal conditions was recently investigated by Casarotti, Monteiro and Penna (2014). Counts of *Sc. thermophilus* and *Bif. animalis* subsp. *lactis* remained stable during the storage period in all treatments, while the counts of *Lb. acidophilus* decreased over time only in the case of the La treatment. Samples fermented by *Bif. animalis* subsp. *lactis* and *Lb. acidophilus* had the highest and lowest sensory attribute scores, respectively, and the scores were correlated to the amounts of lactic and citric acids. Depending on the combination of microorganisms used in fermented milk manufacturing, it had positive or negative impacts on the product's characteristics.

Trigueros and Sendra (2015) investigated conjugated linoleic acid (CLA) and total fatty acids in high-fat, full-fat and low-fat types of several commercial Spanish plain fermented milks (FM) with probiotic bacteria and fermented milk with regular yoghurt cultures. CLA content in fermented milk ranged from non-detected to 93.33 mg/100 g. Neither the fat content of fermented milk nor the presence of the probiotics affected the CLA content (% of total fatty acids). Regarding CLA content per serving size of fermented milk only fat content was positively correlated with CLA.

Tripathi and Giri (2014) studied survival of probiotics during processing and storage of functional foods. The use of microencapsulation, cell protective agents, growth-promoting food ingredients, oxygen barrier packaging materials, antioxidants and modification of storage environments has enabled these microorganisms to survive better in several processes and formulations. As microencapsulation alone results in limited extensions of probiotic viability, a comprehensive approach is required incorporating emerging food processing technologies that may improve and maintain survival of probiotics during processing and storage, along with the recent knowledge on genotypes and expressed traits of probiotics.

9.3.1.1 Exopolysaccharides

Incorporation of probiotic bacteria with ropy or non-ropy starter culture in fermented dairy products has been studied over the last years by many researchers (Girard & Schaffer-Lequart, 2007; Mende et al., 2013; Prasanna et al., 2013). Ropy cultures are capable of producing extracellular polysaccharides (EPS) during the fermentation process, in contrast to non-ropy cultures (Tamime, 2006). Streptococci, lactobacilli and lactococci are some EPS-producing bacteria species which have been successfully used to produce yoghurt with improved physicochemical properties (Prasanna et al., 2013).

The underlying mechanisms of EPS action on milk gel properties are still not fully understood, but have been frequently associated with their structural characteristics (monosaccharide composition, charge, molar mass, degree of branching and chain stiffness) and the molecular interaction with milk proteins (Folkenberg, Dejmek, Skriver, & Ipsen, 2006; Marle, van den Ende, de Kruif, & Mellema, 1999; Marshall & Rawson, 1999; Mende et al., 2013). Some authors observed a positive correlation between viscosity of milk fermented with two strains of *Sc. thermophilus* and the molar mass of EPS (Petry et al., 2003). Charged EPS increased apparent viscosity and the resistance to shear by enhancing the intramolecular repulsion resulting in higher hydrodynamic radius and intrinsic viscosity and via electrostatic interactions between anionic EPS and milk proteins.

Over the last years, EPS-producing *Bifidobacterium* strains as functional starters were used for low-fat yoghurt manufacturing. EPS-producing bifidobacteria could be also used as probiotic strains in milk products exerting their potential health and functional properties at the same time as improving the physicochemical properties of the product, thus have a dual role. *Bifidobacterium longum* subsp. *infantis* CCUG 52486 has already been characterized as a probiotic strain (Gougoulias, Tuohy, & Gibson, 2008; Patel, Michaud, Singhania, Soccol, & Pandey, 2010; Prasanna, Grandison, & Charalampopoulos, 2012). Prasanna et al. (2013) showed that the EPS-producing *Bif. longum* subsp. *infantis* CCUG 52486 was a promising in situ EPS-producing probiotic strain which could be used in combination with traditional yoghurt bacteria to produce low-fat set yoghurt with probiotic activity and improved physicochemical and rheological properties. Also, the milk gelation time is dependent on the kinetics of acidification of the EPS-producing strains (*Lactococcus lactis* subsp. *cremoris*, *Sc.*

thermophilus, *Lb. delbrueckii* subsp. *bulgaricus*) whereas the pH range of gelation depends mostly on the presence of EPS (Girard & Schaffer-Lequart, 2007).

Mende et al. (2013) presented a new approach to investigate the impact of EPS from lactic acid bacteria on the rheological characteristics of acid milk gels by decoupling microbial EPS synthesis from milk acidification. Generally, EPSs can improve technological characteristics of fermented dairy products, like stability and texture, and may also offer protection to cell against phage attack, desiccation and osmotic stress, thus behaving as prebiotics and improve immunity to fight against pathogenic organisms (Ruas-Madiedo, Hugenholtz, & Zoon, 2002). Some authors reported that EPSs have been identified as blood cholesterol-lowering, immunostimulatory, antitumoral and antiulcer agents (Oliveira et al. 2011; Ruas-Madiedo et al., 2002; 2007).

9.3.2 Prebiotics

Inulin is a prebiotic which is used as a non-digestible ingredient beneficially affects humans since it selectively stimulates growth and/or activity of one of limited number bacteria in the colon (Debon et al., 2010). Inulin is a soluble and fermentable fibre named fructan with 2–60 fructose units linked via β (1–2) bond. It is partly soluble in water (10 %, 20 °C) and has a sweetening power of 15 % of sucrose. The extensive use in dairy industry is based on nutritional and technological properties of inulin (Meyer et al., 2011). Not only the dietary fibre properties of inulin are important (such as the positive effect on bowel habit), but also the prebiotic properties (Tungland & Meyer, 2002). These arise from the fact that inulin can cause a specific shift in the composition of the colonic microbiota that has beneficial effects for the human host (Gibson, Probert, van Loo, Rastall, & Roberfroid, 2004). This specific increase in bifidobacteria (the so-called bifidogenic effect) is found in humans of all ages (Meyer & Stasse-Wolthuis, 2006) and it is linked to a variety of beneficial physiological effects. These include improved bowel habits (Marteau et al., 2011), increased calcium absorption with positive effects for bone health (Meyer & Stasse-Wolthuis, 2006), a lowering of serum lipids with relevance for heart health (Brighenti, 2007), a positive effect on feeling of satiety with potential positive consequences for weight management (Cani, Joly, Horsmans, & Delzenne, 2006), a potential effect to enhance resistance to infections (Cummings, Christie, & Cole, 2001) and to stimulate the immune system. Inulin at high intakes (20 g/day) might also decrease plasma triglycerides and cholesterol and might improve glucose tolerance in diabetic.

The technological use of inulin is based on its properties as a sugar replacer (especially in combination with high intensity sweeteners), fat replacer and texture modifier. For fat replacement in low-fat dairy products inulin seems particularly suitable as it may contribute to an improved mouthfeel. Guggisberg, Cuthbert-Steven, Piccinah and Eberhard (2009), Guven, Yasar, Karaca and Hayaloglu (2005), Kip, Meyer and Jellema (2006) and Paseephol, Small and Sherkat (2008) showed

that especially long-chain inulin in addition to low-fat yoghurt resulted in enhanced creaminess. The effects of inulin as prebiotic on physicochemical characteristic, sensory and microbiological characteristics of fermented milk have been investigated by some researchers (Crispín-Isidro et al., 2014; Debon et al., 2010; Meyer et al., 2011; Souza Oliveira, Perego, et al., 2011).

When inulin is added to food in low concentrations, the rheological properties and the sensory quality of the product will not be affected strongly due to the neutral or slightly sweet taste and the limited effect on viscosity of this ingredient (Franck, 2002; Kalyani Nair, Kharb, & Thompkinson, 2010). However, to make inulin-based dietary fibre inulin should be added in amounts that range from 3 to 6 g per 100 g or 100 mL (EC Regulation 2006) and contents of 3–8 g per portion in order to assure its bifidogenic effect (Coussement, 1999; Meyer & Stasse-Wolthuis, 2006). Souza Oliveira, Perego, et al. (2011) also demonstrated that the supplementation of skim milk with inulin, even at a low concentration, significantly improves the growth and viability of *Lb. acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium lactis* in non-fat fermented milk.

In addition to inulin, lactulose is also used in food industry mainly as prebiotic. It is a synthetic disaccharide obtained by isomerization of lactose, which is present in milk and whey in relatively high content, approximately 4.5 % as an average (Zokaee, Kaghazchi, Zare, & Soleimani, 2002), and contains fructose instead of glucose. Since it is not absorbed in the small intestine, it has the potential to function as a prebiotic (Kontula, Suihko, von Wright, & Mattila-Sandholm, 1999). Moreover, several studies showed the effectiveness of the lactulose to stimulate the growth of bifidobacteria (Olano & Corzo, 2009; Sako, Matsumoto, & Tanaka, 1999; Shin, Lee, Pestka, & Ustunol, 2000). Lactulose appears as an important food ingredient for the production of functional foods and effects on the acidification kinetics, post-acidification, growth and metabolism of binary co-cultures of *Lb. bulgaricus*, *Lb. acidophilus*, *Lb. rhamnosus* and *Bif. lactis* with *Sc. thermophilus*, as well as on their survival during skim milk fermentation (Souza Oliveira, Rodrigues Florence, Perego, & Converti, 2011). Additionally, milk solid supplementation is a good practice to improve probiotic growth during the fermentation period and favour bacterial viability in the product (Marafon et al., 2011).

Different starter cultures and functional food ingredients (prebiotics, milk proteins, whey protein concentrate) incorporated in fermented milks significantly affects the composition, flavour, texture and other sensory characteristics, as well as viability of microorganisms of the final products.

9.3.3 Kombucha

Novel researches are dealing with the possibilities of the application of kombucha as non-conventional starter for milk fermentation (Hrnjez, Vaštag, et al., 2014; Hrnjez, Vukić, et al., 2014; Malbaša, Milanović, Lončar, & Kolarov, 2009; Milanović et al.,

2008, 2012; Vukic et al., 2014). Kombucha is a symbiotic association of yeast (*Pichia*, *Zygosaccharomyces*, *Saccharomyces*, *Schizosaccharomyces*, *Saccharomycodes*, *Torulaspora*, *Candida* and *Brettanomyces*,), acetic acid bacteria (*Acetobacter* and *Gluconobacter*) and lactic acid bacteria (*Lactobacillus* population). Traditionally, kombucha is cultivated on dark and green tea, but it can also be cultivated on a dark beer, red wine, whey and lactose. Kombucha tea beverage, as a consequence of fermentation, contains ethanol, carbon dioxide, a high concentration of acid (gluconic, acetic and lactic) as well as a number of other metabolites and is thought to contain a number of health-promoting components (Battikh, Bakhrouf, & Ammar, 2012; Malbaša et al., 2009). Marsh, O'Sullivan, Hill, Ross and Cotter (2014) have carried out the first culture-independent, high-throughput sequencing analysis of the bacterial and fungal populations of five distinct pellicles as well as the resultant fermented kombucha at two time points. They found that the major bacterial genus present was *Gluconacetobacter*, present at >85 % in most samples, with only trace populations of *Acetobacter* detected (<2 %). A prominent *Lactobacillus* population was also identified (up to 30 %), with a number of sub-dominant genera, not previously associated with kombucha, also being revealed. The yeast populations were found to be dominated by *Zygosaccharomyces* at >95 % in the fermented beverage, with a greater fungal diversity present in the cellulosic pellicle, including numerous species not identified in kombucha previously.

Malbaša et al. (2009) investigated the possibility of manufacturing of fermented dairy product using different concentrations of evaporated kombucha inoculum. They concluded that fermentation time was similar at 10–15 % of kombucha inoculum, but the process was two times faster with yoghurt starter. Iličić, Kanurić, Milanović and Malbaša (2012) focused on fermentation of lactose from a model system (black tea) and from two types of milk (0.9 % w/w and 2.2 % w/w of fat) by application of kombucha. Quantities of the applied kombucha starter were of 10 % (v/v) and 15 % (v/v). Analysis of the fermentation curves showed that inoculum concentration (10 % v/v or 15 % v/v) did not significantly affect the rate of fermentation. The fermentation time for the samples with 2.2 % w/w of fat was slightly shorter than that for low fat.

The possibility of application of kombucha cultivated at two different tea types in combination with probiotics for milk fermentation at different temperatures (37 and 42 °C), and the physicochemical and textural properties of the so-manufactured fermented dairy products were assessed (Milanović et al., 2012). The combination of probiotic starter culture and kombucha inoculums cultivated at *Camellia sinensis* (black tea) and *Thymus serphyllum* (thyme tea) were used for milk fermentation. The results showed that kombucha inoculums cultivated at different tea types in combination with probiotic starter culture could be successfully used to produce new types of fermented milk products.

The effect of kombucha starter culture on rheological properties, texture and microstructure of milk during fermentation as well as on protein profile was investigated by Vukic et al. (2014). The results were compared with the samples obtained with yoghurt starter culture and probiotics. Quality parameters were measured during the fermentation at 42 °C at pH of 5.4, 5.1, 4.8 and 4.6. The obtained results of

viscosity at pH = 5.4 revealed that gelation process was similar to the samples produced with kombucha and probiotics. The samples produced with kombucha had the highest complex modules that caused better rheological characteristics of the final product. Protein profile analysis revealed more stable α and β casein fractions as compared to other protein fractions of fermented dairy products obtained with kombucha starter culture at different fermentation temperature during milk fermentation (Hrnjez, Vukić, et al., 2014). Although the fermentation time of probiotic yoghurt is two times shorter than kombucha fermented milk production, application of kombucha in fermented dairy productions is justified by nutritional and technological aspects of view. The results of viscosity, textural characteristics, nutritive and sensory properties of kombucha fermented milk products showed that it is a novel high nutritive value fermented milk product (Hrnjez, Vaštag, et al., 2014; Hrnjez, Vukić, et al., 2014; Iličić et al., 2013; Milanović et al., 2012).

9.3.4 Transglutaminase

The attempts have been made to improve low-fat yoghurt gel properties by adding transglutaminase in combination with different ingredients (glutathione, milk proteins or whey protein concentrates) or application of sophisticated techniques (high-pressure homogenization, high-pressure processing, ultrasound and pulse electric field).

From 1990s numerous studies explored the potential applications of enzyme transglutaminase (TGase, EC 2.3.2.13) in food protein systems, especially milk proteins. Transglutaminase is an enzyme which can modify the heat stability, solubility, hydration, emulsifying, rennetability, gelation and rheological properties of a variety of food proteins. This enzyme catalyses the acyl transfer reaction between γ-carboxyamide groups of peptide-bound glutamine residues and the ε-amino groups of lysine residues, leading to the formation of intra- and intermolecular isopeptide bonds. The enzyme reaction results in the formation of covalently cross-linked protein polymers. Accordingly to that, such reactions can improve the structure and functionality of protein systems. The macro-molecular structure of individual proteins determines the rate of TGase cross-linking. Milk proteins—caseins and whey proteins—individually are good substrates for TGase. In a complex system such as milk, the caseins are cross-linked preferentially over the native whey proteins. Whey proteins require structural modification, e.g. heat-induced denaturation, to allow their participation in cross-linking reactions, either between their fractions or with caseins (Ikura, Yoshikawa, Sasaki, & Chiba, 1984; Iličić et al., 2008; Iličić, Milanović, & Carić, 2015; Milanović et al., 2007; Motoki & Seguro, 1998; O'Sulivan, Kelly, & Fox, 2002). The hydrophilic part of the k-casein known as caseinomacropeptide (CMP) is highly reactive towards TGase action. In contrast, whey proteins in their native structure are less prone to the cross-linking reaction mainly due to their globular conformation stabilized by disulphide bonds (Bönisch, Lauber, & Kulozik, 2004; Færgemand, Sørensen, & Jørgensen, 1999; Tolkach & Kulozik, 2005).

Potential benefits of TGase treatment on milk proteins including physical stabilization and structural modification as well as physicochemical, textural and rheological properties of fermented dairy products, particularly yoghurt with a reduced fat content, have been extensively studied over the last years. Enzyme TGase is commonly used as commercial microbiological preparation isolated from the *Streptoverticillium* strains purchased from Ajinomoto Co. Inc. (Japan). There are two different ways for the application of TG in yoghurt manufacturing—either simultaneously with starter culture or prior to the fermentation (Bönisch et al., 2004; Bönisch, Huss, Lauber, et al., 2007; Bönisch, Huss, Weitl, et al., 2007; Bönisch, Lauber & Kulozik, 2007; Iličić et al., 2013, 2014; Lorenzen, Neve, Mautner, & Schlimme, 2002; Milanović et al., 2007; Ozer, Kirmaci, Oztekin, & Atamer, 2007; Rodriguez-Nogales, 2006). In the case when TGase is used together with the starter culture, the enzyme is gradually inactivated upon acidification. Pretreatment of milk with TG prior to fermentation requires additional process time as well as a thermal inactivation step, but has the advantage of a constant pH during the cross-linking reaction, thus offering a wider range of possible incubation condition. In most cases, the TGase pre-treatment is followed by a heating step to inactivate the enzyme at 80 °C for 1 min (Lorenzen et al., 2002; Milanović et al., 2009; Ozer et al., 2007). Færgemand et al. (1999) attributed this effect to a reduced availability of low molecular mass peptides for the growth of lactic acid bacteria. Generally, heat treatment of milk prior to cross-linking is necessary because the reactivity of TGase is very low in unheated or pasteurized milk, despite the high reactivity of the casein (Rodriguez-Nogales, 2006). Several authors suggested TGase concentrations in the range of 9–14 U g^{-1} protein to improve the physical, chemical and sensory properties of set style skim milk yoghurt in comparison with untreated samples (Lorenzen et al., 2002; Ozer et al., 2007). According to Farnsworth, Li, Hendricks and Guo (2006), a fourfold higher TGase concentration was necessary to obtain the increased viscosity in stirred yoghurt in comparison with set style yoghurt. In contrast, the application of transglutaminase preparation containing glutathione (GSH) resulted in significantly higher apparent viscosity levels compared with TGase without GSH, even at low enzyme concentrations of 0.6–1.0 U g^{-1} protein (Bönisch, Huss, Weitl, et al., 2007).

Protein fortification of milk has an important role on the reaction of TGase. Protein ingredients such as milk powder, WPC and sodium caseinates are commonly used to fortify the protein content of milk in order to obtain the desired yoghurt structure. Addition of TGase at a minimal level of 0.01 % (w/w) contributed significantly to the formation of low-fat stirred probiotic yoghurt with improved physical characteristics and better textural properties, while higher concentrations of TGase (0.02–0.05 % (w/w)) produced even more favourable effects. Simultaneous addition of TGase (0.01 % (w/w)) and WPC (0.3 % (w/w)) decreased the yoghurt's textural properties than those of the yoghurt prepared with TGase or WPC alone. It is very probable that an increased rate of casein cross-linking in the presence of TGase prevailed over the rate of formation of whey proteins aggregates, giving a non-uniform structure of gel matrix and, therefore, a coarse and lumpy product with poor texture (Milanović et al., 2009).

The ratio of casein to whey protein affects the mesh size of the resulting yoghurt gel network (Bönisch, Huss, Lauber, et al., 2007). A decrease of this ratio increases the maximum gel strength of the yoghurt. Also, Bönisch et al. (2004) found that apparent viscosity and degree of protein polimerization (DPP) of stirred yoghurt produced with TGase increased with protein fortification (from 3.4 to 4.4 % protein content).

Viscosity and the gel structure, besides mentioned factors, are influenced by the type of starter culture applied in yoghurt processing. The impact of kombucha starter with or without added TGase on rheology, textural properties and microstructure of fermented dairy products, in comparison to probiotic starter culture, is published recently (Iličić et al., 2013). The addition of TGase in milk increased approximately 10.5 % hysteresis loop area, 39 % firmness and 48 % consistency in probiotic yoghurt and had more firm and stable gel structure than kombucha fermented dairy product. The protein network in kombucha fermented milk products was broken in small aggregates and contained fewer void and interstitial spaces compared with fermented milk produced with probiotic starter. The microstructure of both samples with TGase was a more regular and dense protein network than in samples without TGase (Fig. 9.1).

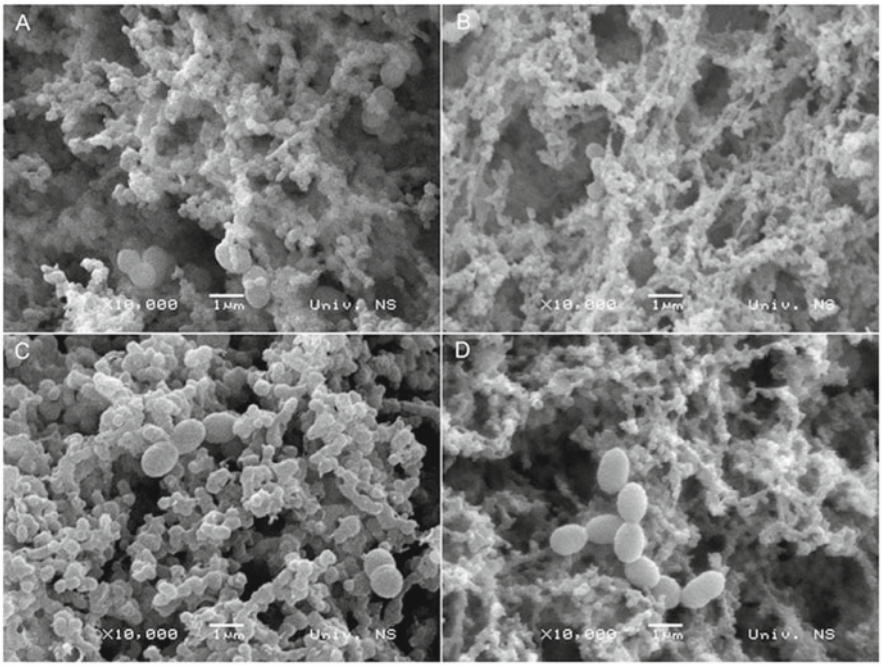

Fig. 9.1 Microstructure of fermented milk products manufactured with different starters and with and without TGase cross-linking prior to inoculation: (**a**) PFM (probiotic fermented milk); (**b**) PFM + TG (probiotic fermented milk with transglutaminase); (**c**) KFM (kombucha fermented milk); (**d**) KFM + TG (kombucha fermented milk with transglutaminase) (Iličić et al., 2013)

9.4 Application of Novel Techniques

Non-thermal food preservation processes represent an alternative to conventional thermal processing. Among these new preservation methods, high-pressure processing (HPP), high-pressure homogenization (HPH), ultrasonic processing (USP) and pulse electric field (PEF) can be successfully applied alone or together with other preservation methods.

High-pressure processing was first investigated in the late nineteenth century by Hite (1899). He found that high-pressure treatment of milk resulted in the destruction of spoilage microorganisms and the inactivation of enzymes, and therefore markedly increased its shelf-life. Engineering aspects of high-pressure processing were extensively investigated by Bridgman in the early twentieth century (Bridgman, 1964). High-pressure processing is a "non-thermal" food preservation technique that inactivates harmful pathogens and vegetative spoilage microorganism by using pressure rather than heat. HPP uses intense pressure (about 400–600 MPa) at chilled or mild process temperatures (<45 °C), allowing most foods to be preserved with minimal effects on taste, texture, appearance or nutritional value (Balasubramaniam, Farkas, & Turek, 2008).

Generally, HPP treatment reduces the size of casein micelles through solubilization of colloidal calcium phosphate (Huppertz, Fox, & Kelly, 2004a). However, HPP-induced interactions with denatured whey proteins tend to increase micelle size and the net effect, after treatment under certain conditions, may be either an overall increase or a net reduction in size (Huppertz, Fox, & Kelly, 2004b, 2004c).

It has been demonstrated that HPP treatment of the milk base used for the production of yoghurt alters the quality parameters of the final product (Hemar, Liu, Meunier, & Woonton, 2010; Huppertz, Smiddy, Upadhyay, & Kelly, 2006; Lanciotti, Vannini, Pittia, & Guerzoni, 2004).

There have been various claims in the literature about the applicability of HPP for improving the properties of yoghurt. In traditional yoghurt manufacture, milk is heated (typically at 90 °C for 10 min) and cooled to ~45 °C prior to the addition of cultures. The heat treatment denatures the whey protein and increases its water binding properties. It is well known that the heat treatment is required to build viscosity in yoghurts. Heat treatment also reduces gelation time and increases gelation pH of acid gels. Thickeners and stabilizers are usually also added to produce gels of acceptable texture and to avoid syneresis, especially in low-fat products (Anema, Kim Lee, Lowe, & Klostermeyer, 2004; Udabage, Augustin, Versteeg, & Kelly, 2010).

HPP is an alternative method of modifying milk proteins towards improving the yoghurt-making properties of milk which are related to the formation of a tight and compact structure. The HPP treatment of milk also enables the inactivation of its pathogenic and spoilage microflora, while minimally affecting its endogenous enzymes and its quality characteristics and nutritional value. HHP treatment can cause changes in both caseins and whey proteins. Previous studies on HPP implementation in yoghurt manufacture have shown that HPP-treated milk exhibits higher

rate of acidification and coagulates at higher pH values; as a result, yoghurt prepared from HPP-treated milk exhibits lower amounts of whey separation and increased gel strength compared to those made from non-pressuresed milk.

The effect of high hydrostatic pressure treatment of a milk base in the absence or presence of a transglutaminase protein cross-linking step on the flavour development of yoghurt were investigated by Tsevdou et al. (2013). The HPP of milk increased significantly the volatile compound formation rate whereas it did not affect the duration of the lag phase of formation, with the exception of acetaldehyde and diacetyl formation.

HPP has been proposed as a method that could contribute to texture improvement of dairy products. When HPP is used to improve the textural attributes of dairy products at their final structure, the common processing conditions ranged from 100 to 400 MPa and from 10 to 25 °C for process times in the range of 10–15 min. According to previous studies, a pressure range of 200–300 MPa and a temperature range of 20–25 °C for process time of 10 min can lead to a final product with significant improved texture and acceptable sensory characteristics, with limited inactivation of yoghurt bacteria (Ancos, Cano, & Gómez, 2000; Krompkamp, Moreira, Langeveld, & Van Mil, 1995; Tanaka & Hatanaka, 1992; Tsevdou & Taoukis, 2011).

Yoghurts made from high-pressure-treated skim milk show an improved creamy texture compared to traditional yoghurts (Ciron, Gee, Kelly, & Auty, 2012; Udabage et al., 2010). Udabage et al. (2010) investigated physical properties of stirred yoghurt made from reconstituted skim milk that was high-pressure-treated at 100, 250 or 400 MPa, at 25, 70 or 90 °C, for 10 min, prior to inoculation with yoghurt cultures. High-pressure treatment of skim milk at 25 °C, before or after heat treatment, gave stirred yoghurts of similar viscosities to that made from conventionally heat-treated milk. Combined use of thermal treatment (85 °C, 30 min) and high hydrostatic pressure (300–676 MPa, 5 min) assures extensive whey protein denaturation. Although reaggregation of casein submicelles occurs during fermentation, the net effect of combined high hydrostatic pressure is the improvement of yoghurt yield stress and reduction of syneresis (Harte, Luedecke, Swanson, & Barbosa-Cánovas, 2003). The application of high hydrostatic pressure (5 min at 676 MPa) combined with thermal treatment (85 °C for 30 min) resulted in yoghurt gels with attractive physicochemical characteristics and high water-holding capacity (Penna, Rao-Gurram, & Barbosa-Canovas, 2007).

High-pressure homogenization, in which pressures of up to 400 MPa are used, has recently emerged as a process for achieving more uniform fat globule size and partially denaturing whey proteins, as well as inactivating enzymes and microorganism (Huppertz, 2011).

Serra, Trujillo, Quevedo and Ferragut (2007) reported that yoghurts made from milk processed by HPH at 200 or 300 MPa had higher gel firmness, less syneresis and lower titratable acidity than those obtained from conventionally treated milk with skim milk powder. Another study by this group showed that the application of HPH of milk instead of the conventional thermal treatment may affect the flavour quality of the final products proportionally to the pressure conditions (Serra, Trujillo, Guamis, & Ferragut, 2009).

The HPH treatment seems to favour the growth of *Streptococcus thermophilus* with respect to that of *Lactobacillus delbrueckii* subsp. *bulgaricus*, regardless of the level of pressure applied (Lanciotti et al., 2004).

Ultrasound technology is based on mechanical waves at a frequency above the threshold of human hearing (>16 kHz). Results have shown that ultrasounds, already applied for food processing and preservation (e.g. emulsification, homogenization, extraction, freezing), can induce changes in the physical properties (e.g. viscosity, water binding capacity) of biopolymers such as pectin, starch and proteins (McClements, 1995) to obtain ingredients or semi-manufactured products with tailored functional characteristics. Such effects are due to the cavitation phenomenon, which is the spontaneous formation and collapse of bubbles that leads to the generation of local extreme temperatures and pressures, which in turn produce intense shear energy waves and turbulence in the vicinity of the material (Anese, De Bonis, Mirolo, & Ruocco, 2013; Barbosa-Canóvas & Rodríguez, 2002; Mason, 1998).

Having in mind that ultrasonication can pasteurize and preserve foods by inactivating many enzymes and microorganisms at mild temperature conditions, which can improve food quality in addition to guaranteeing stability and safety of foods (Chandrapala, Oliver, Kentish, & Ashokkumar, 2012), ultrasound treatments might improve fermentation processes of milk due to enhancement of membrane permeability of dairy cultures, allowing the release of intracellular enzyme such as β-galactosidase out from the cell (Ewe, Wan Abdullah, Bhat, Karim, & Liong, 2012).

Chandrapala, Zisu, Kentish and Ashokkumar (2013) have also reported on the effect of sonication on the chemically induced gelation of micellar casein systems. In cheese and yoghurt manufacturing, chemically induced gelation process is a critical step. In order to see if sonication of casein systems affects the gel properties of casein micelles, experiments were carried out on 5 wt% micellar casein solution. As shown in, sonication of micellar casein solution prior to the addition of tetra sodium pyrophosphate (gelation agent) led to the formation of a firmer gel with a significant decrease in syneresis. This study has demonstrated that sonication can be an effective technique to alter the functionality of dairy systems and one should take care of the processing sequence.

The effect of ultrasound on the physical properties of yoghurt has been studied by Wu, Hulbert and Mount (2001) and Vercet, Oria, Marquina, Crelier and Lopez-Buesa (2002). The former authors examined the effect of ultrasound on milk homogenization and determined and evaluated the effect of ultrasound on the pH change during yoghurt fermentation, and water holding capacity, viscosity and syneresis of the produced yoghurt. USP was carried out in a water bath at 15 °C to reduce thermal effects, and all milks were heat treated (95 °C for 10 min) prior to USP. At the highest power level used, USP produced much smaller fat droplets than the conventional homogenization control treatment (12.4 MPa at 60 °C). Increasing the ultrasound amplitude level before inoculation significantly improved the water holding capacity and viscosity and reduced syneresis. Another study (Vercet et al., 2002) showed that the simultaneous application of heat (40 °C) and ultrasound (12 s at 20 kHz) under moderate pressure (2 kg cm^{-2}) improved the rheological properties

of yoghurt by still unknown mechanisms. Although measurements on intact and slowly stirred samples showed that treated yoghurts had stronger structures, it was also proved that the homogenization of milk fat globules by means of a manothermosonication (MTS) treatment was not the cause of the textural differences observed. Further research should be done to evaluate the contribution of longer fermentation times and the denaturalization of proteins to the increased consistency and viscosity of MTS yoghurts (Soria & Villamiel, 2010).

Two studies reported that the USP treatment of skim milk before acidification (by cultures) produced gels with higher firmness, shorter gelation time, higher gelation pH, better water-holding capacity and better sensorial properties than those produced from conventionally heated skim milk (Fig. 9.2) (Riener et al., 2010; Riener, Noci, Cronin, & Lyng, 2009).

Nguyen and Anema (2010) examined how thermal and non-thermal effects of USP affected the properties of USP-treated skim milk and acid gels produced from it. USP without temperature control resulted in the generation of considerable heat, with the milk reaching ~95 °C within 15 min of treatment, which completely denatured whey proteins. The changes to the casein micelle size were observed, with decreases during the early stages of US and increases (because of aggregation) on prolonged treatment. Significant k-casein dissociated from the micelles. Acid gels prepared from these ultrasonicated samples increased in firmness (final G') up to a maximum final G' after ~15 min of US, followed by a decrease from this maximum on prolonged treatment. The authors concluded that, in skim milk, most of the effect of USP can be related to the heat generated from the treatment, with USP itself having only a small effect on the milk when the temperatures are controlled.

Pulsed electric field processing involves the application of very short, high voltage pulses to a food product, which is placed between or passed through two electrodes. The intensity of electric fields varies between 15 and 50 kV/cm although it lasts only for some seconds (Ravishankar, Zhang, & Kempkes, 2008). PEF in combination with sub-pasteurization temperatures has the potential of achieving

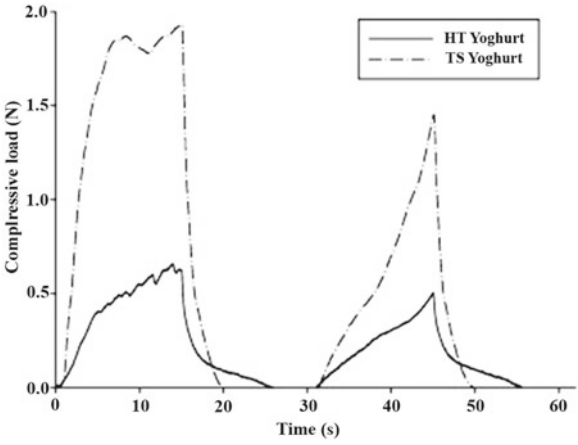

Fig. 9.2 Typical texture profile analysis (TPA) for thermosonication (TS) and conventionally produced (HT) yoghurts (1.5 % fat) (Riener, Noci, Cronin, & Lyng, 2010)

microbial inactivation levels in milk equivalent to conventional thermal pasteurization (Shamsi, Versteeg, Sherkat, & Wan, 2008; Walkling-Ribeiro, Noci, Cronin, Lyng, & Morgan, 2009). When used in the production of dairy products, PEF affects the size of the casein micelles and influences in the coagulation of casein (Cruz, Faria, Saad, & Cristianini, 2010). The intensity, duration and temperature of PEF treatment of milk directly affects the structures of casein micelles and whey proteins and demonstrably alters the functional properties such as aggregation, hydrophobicity, thermal stability, rennetability, gelation rate, gel strength and emulsion stability of milk protein constituents (Buckow, Scott Chandry, Ng, & Swanson, 2014; Eberhard & Sieber, 2006). Dunn and Pearlman (1987) reported the extension of shelf-life of yoghurt and the possibility of semi-selective inactivation of yeasts in yoghurt by PEF processing. Yoghurt-based product using pasteurized milk as raw material in combination with thermal treatment/PEF (60 °C/30 s and 30 kV/cm of pulse intensity for 32 μs) did not have its sensorial quality affected. In addition, this product was shown to have a decreased growth of mesophylic aerobic microorganisms and molds and yeasts (Yeom, Evrendilek, Jin, & Zhang, 2004).

9.5 Functional Characteristics and Health Benefits

Following the current demands world widely, investigations of dairy industry have been aimed undertaken to determine the effects of fermented milks on humans and animals. Several principal health effects are studied so far and could be divided into two groups. The first group refers to “nutritional function”, which means function of supplying more nutrition efficiently. The second group is “physiological function”, which includes the therapeutic functions beyond nutritional effects (Cifelli, German, & O’Donnell, 2011).

9.5.1 Antioxidative Activity

Bioactive components are constituents in food or dietary supplements that meet basic nutritional needs. They are responsible for health changes and are capable of eliciting a biological response, such as reducing hypertension, stimulation of the immune system preventing cancer, enhancing lean body mass, or killing bacteria (Siro, Kapolna, Kapolna, & Lugasi, 2008). Nowadays, bioactive peptides have gained increasing attention for human health promotion and deferment of age-related diseases. Recent research has focused on naturally occurring bioactive components in fermented dairy products, such as bioactive peptides (Korhonen, 2009; Takano, 2002; Urista, Fernández, Rodriguez, Cuenca, & Jurado, 2011). Beneficial health effects of yoghurt are partly linked to these bioactive peptides as products of a proteolysis, which occur during fermentation and storage. Application of the different starter cultures in single or multiple-strain, such as bacteria, yeasts, molds or

combination of these microorganisms offer a wide range of possibilities to reproduce different health aspects with new bioactive components. Next to bioactive peptides, other interesting ingredients such as free amino acids (FAAs lactoferrin, CLA micronutrients, sphingolipids or exopolysaccharides) are also retrieved from the milk. Milk-derived bioactive peptides lead to several important health-promoting activities, such as anti-hypertensive, anti-microbial, anti-oxidative, immune-modulatory, opioid and mineral-binding properties (Korhonen, 2009; Samaranayaka & Li-Chan, 2011; Udenigwe & Mohan, 2014).

Nowadays, plants, fruits and spices have a great attention as natural sources of nutraceuticals and in particular antioxidants since they contribute to the protection of cells from oxidative stress and preserve them from severe diseases (Aliakbarian, Palmieri, Casazza, Palombo, & Perego, 2012; Halah & Nayra, 2011). Therefore, numerous studies have been focused to combine healthy and functional characteristics of both, natural antioxidants (as ingredients) and fermented dairy products, especially yoghurt (as nutritious food) with the aim to obtain novel further functional and consumer-acceptable products. Even though phenolic compounds are present in ruminant milk, several production factors (such as pasteurization) have been reported to reduce the content of the phenols in dairy products (O'Connell & Fox, 2001). The addition of the herb extracts, fresh spices or fibres from fruit by-products have contributed to the development of the milk and yoghurt products containing health-promoting substances such as phytochemicals. Although investigations of enhanced dairy products with phenolic compounds have been started in the past decade, up to date only inconsiderable number of the investigations have been published.

The production of the low-fat fermented milk with the addition of green bell pepper juice in a concentrations of 1, 2 and 3 %, as a source of antioxidants compounds (mostly phenols, flavonoids and carotenoids) fermented by combination of lactic acid bacteria (LAB) (2 %) and probiotics strains *Lb. rahamnosus* (2 %) have been shown as a promising methods for the production of fortified fermented products (Halah & Nayra, 2011). The increased concentration of pepper juice increased radical scavenger activity (RSA %), total phenols (TPC) and carotenoids of both, fresh and stored fermented dairy samples. Also, this supplementation enhanced the growth rate of *Lb. rhamnosus* while decreased the growth of lactic acid bacteria. The RSA (%) of fermented milk supplemented with bell green pepper juice and inoculated with *Lb. rhamnosus* and LAB starter during cold storage varied from 52 % to 72 % with 1 % and 3 % added extract, respectively. The higher antioxidative activity is not only the result of added juice, but more likely of LAB proteolitic activity and realized bioactive peptides as well as oxygen radicals system of the cell-free extracts of the dead cultures which increase during storage. The addition of pepper juice has no hazard or inhibition effect on viability of this strain while it could inhibit growth of other microorganisms such as yeasts and molds.

Recent research has showed possibility of production novel functional fermented dairy products enhanced with phenolic extracts from olive and grape pomace (Aliakbarian et al., 2014). The milk fermentation was obtained by using co-culture of two different microorganisms *Sc. thermophilus* (TA040) and the probiotic *Lb.*

acidophilus (LAC4). Results of this research showed that phenolic compounds are stable during the fermentation. Even total phenol content of all fortified samples increased; its trend was dependent on storage time. The content of total phenol in the samples after 7 days of storage was 92.27 ± 3.06 mg CAE/g and 98.59 ± 4.89 mg CAE/g in the samples with grape and olive pomace, respectively. The obtained concentrations are almost 50 g (four soup-spoonfuls) of olive oil containing 200 mg/kg of total polyphenols pomace. In contrast to the number of viable cells of *S. thermophilus*, which decreased during the first 14 days of storage, count of *L. acidophilus* remained almost constant in all fermented milks. This research also showed functional effect of added extracts though the increment of radical scavenger activity. Thus, natural extracts enhanced yoghurt may have the potential to serve as enhanced functional yoghurt with significant health benefits.

The supplementation of fermented milk with Neem (*Azadirachta indica*), traditional medicine used in Asia, gave also great promises to the concept of novel functional fermented dairy products. Shori and Baba (2013) produced this type of enhanced yoghurt with the significant antioxidative activity and higher content of the phenols in comparison to the plain yoghurt. In addition, the antioxidative potential of *A. indica*-yoghurt showed anti-diabetic activity (i.e. against α-amylase and α-glucosidase). The antioxidant capacity and total phenolic content (TPC) of samples increased during the storage. Enhanced yoghurt with *A. indica* had highest TPC (74.9 ± 5.1 lgGAE/mL; $p < 0.05$) on day 28 and DPPH inhibition (53.1 ± 5.0 %; $p < 0.05$) on day 14 compared to control plain yoghurt (29.6 ± 1.1 lgGAE/mL and 35.9 ± 5.2 %, respectively).

There have been other researches focused on developing novel probiotic yoghurt, supplemented with different spices as a source of functional flavouring agents including those with antioxidant, antimicrobial, anti-inflammatory and anticarcinogenic characteristics. Different combinations of probiotics with spices may provide better therapeutic properties, but antimicrobial effect of spices on probiotic viability should be considered.

The functional probiotic yoghurt containing selected spice oleoresins (cardamom, cinnamon and nutmeg) with acceptable sensory therapeutic levels of probiotics and with beneficial antioxidant capacity was developed by Illupapalayam, Smith and Gamlath (2014). Different probiotic yoghurts were obtained by selected starter cultures *Lb. acidophilus* strains 5(LA5) or *Bif. animalis* subsp. *lactis* (BB12) in combination with cardamom, nutmeg and cinnamon. Produced yoghurts had acceptable counts of viable probiotics. Furthermore, significant increase in survival of both starters and increased antioxidant activity after 28 days of storage was noticed. In the group of yoghurt products obtained by LA5, the highest antioxidant activity on DPPH radicals was recorded for cardamom-yoghurt on the first day of storage (79.5 %) followed by nutmeg and cinnamon-yoghurt (69 %) and control-yoghurt (57 %). In the group of yoghurt products containing BB12, the highest antioxidant activity was recorded at the first day of storage for nutmeg (70 %) followed by cardamom (57 %) and cinnamon-yoghurts (57 %). Cardamom-yoghurt showed the highest antioxidant activity at all storage periods (LA5 or BB12) compared to other spice and control-yoghurts. Overall, acceptability scores for the nutmeg and

cinnamon-yoghurts were not significantly different from the control, but cardamom-yoghurts containing LA5 was significantly different from the other yoghurts. There was no significant difference among nutmeg-, cinnamon- and the control-yoghurt in yoghurts produced by BB12. Nevertheless, cardamom-yoghurt was significantly different suggesting that compared to other types cardamom-yoghurt was well accepted by the consumers.

Some authors investigated the effects of peppermint (*Mentha piperita*), dill (*Anethum graveolens*) and basil (*Ocimum basilicum*) on yoghurt production and its antioxidative activity. They used starter culture containing *Sc. thermophilus*, *Lb. acidophilus*, *Lb. bulgaricus* and *Bifidobacterium bifidum* and herbal water extract for milk fermentation. All these new herbal-yoghurts had higher antioxidant activities than control-yoghurt at the end of fermentation and also throughout the tested 28 days of storage (Amirdivani & Baba, 2011). The highest TPC was found in yoghurt with *A. graveolens* (47.7–1.6 mg GEA/mL) followed by *M. piperita* yoghurt (37.5–2.6), *O. basilicum* yoghurt (28.6–2.1) and control-yoghurt (without herbs) (19.5–1.5 mg GEA/mL). All enhanced yoghurts had the highest values of antioxidative activity and DPPH radicals on the 7th day of refrigerated storage: 58.7 ± 3.9, 52.3 ± 1.8, 45.2 ± 4.4 and 33.6 ± 0.8 % for *A. graveolens*-, *M. piperita*-, *O. basilicum*- and control-yoghurts, respectively. Therewithal, the presence of herbs extends unique sensory properties to the yoghurt due to the specific aromatic compounds. In comparison to the control-yoghurt, *M. piperita*-yoghurt was considered as the best for aroma and overall sensory performance, whereas flavour score was the highest for *A. graveolens*-yoghurt, while it was the lowest for *O. basilicum*-yoghurt. An inhibition activity of herb ingredients on the involved starter culture was not observed.

In addition to the fortification of fermented milks by medical plants in order to obtain new products with better antioxidative and sensory characteristics, utilization of milk from different species (goat, sheep, buffalo, camel, etc.) could also improve functional properties of final products. Significantly higher digestibility and greater antioxidant activity of camel milk α-lactalbumin compared to bovine whey proteins was also observed in the previous study (Salami, Yousefi, Ehsani, & Moosavi-Movahedi, 2009).

The utilization of non-conventional, functional starter cultures holds great promises to manufacture various yoghurts with desirable technological, nutritional and beneficial health advantages (Tripathi & Giri, 2014). Recent studies presented the technological potential of kombucha as an innovative starter culture in dairy industry. Several research teams investigated the influence of kombucha non-conventional starter and processing conditions on antioxidative potential of kombucha fermented milk products. Malbasa, Vitas, Loncar and Milanović (2014) investigated the antioxidant activity of fermented milk products obtained from milk containing 0.8, 1.6 and 2.8 % milk fat at different fermentation temperatures of 37, 40 and 43 °C, by kombucha starter produced on sweetened wild thyme extract. Optimum processing conditions in terms of antioxidant activity for these new products were: milk fat of 2.8 % and fermentation temperature—37 °C. On the contrary, according to predicted built model, in order to obtain products with high sensory quality, these

conditions were: 1.1 % milk fat and process temperature of 43 °C. These results suggest that the processing conditions have significantly greater influence on the values of antioxidative activity on DPPH and OH radicals as well as vitamin C in final products. The same authors also investigated the antioxidative activity of fermented milk products obtained by kombucha fermented on the sweetened stinging nettle extract and sweetened winter savoury extract, under the same conditions as previous experiments (milk with 0.8, 1.6 and 2.8 % milk fat and fermentation temperature 37, 40 and 43 °C) (Vitas, Malbaša, Grahovac, & Lončar, 2013). Kombucha fermented dairy products with stinging nettle (KSN) and with winter savoury (KWS) showed the same antioxidant response to hydroxyl and different response to DPPH radicals. The optimum processing conditions in terms of antioxidant activity were: milk fat around 2.8 % and process temperature of 41 and 43 °C for KSN and KWS, respectively.

Hrnjez, Vaštag, et al. (2014) analysed the effects of kombucha inoculum as a starter culture for milk fermentation and compared with the fermented dairy products obtained by commercial probiotic (ABT-10) and yoghurt (YF-L812) starter cultures during 14 days of storage. The DPPH radical scavenger activity (RSA) in kombucha sample was significantly higher ($P>0.05$) after production (17.88 ± 0.17 %) than in yoghurt and probiotic samples (9.46 ± 0.3 and 13.23 ± 0.04 %, respectively). Kombucha starter used for milk fermentation improved DPPH RSA during refrigerated period due to its own antioxidative capacity. The high content of vitamin C in kombucha product during the storage contributed to the antioxidant activity of the fermented dairy product (Fig. 9.3). Furthermore, significant correlation between the highest values of DPPH antioxidant activities and the quantity of vitamin C in all samples was recorded. In contrary to the DPPH radical's scavenger activity which was below 18 %, ABTS radical cations, RSA of all samples were between 36 and 55 %. Considering the significant biological value of kombucha tea proved previously by different research groups, fermented dairy product obtained by kombucha inoculum could be appropriate novel product which could increase assortment of functional food.

The search for bioactive peptides is intensifying because of the potential risks associated with the use of synthetic therapeutics. Thus, peptide liberation by applied starter culture has received a great attention. However, proteolytic activity of *Lb. acidophilus* (ATCC_4356™), *Lactobacillus casei* (ATCC_393™) and *Lactobacillus paracasei* subsp. *paracasei* (ATCC_ BAA52™) in yoghurt were tested for antioxidant and antimutagenic activities (Settachaimongkon et al., 2014). The degree of proteolysis highly correlated with these bioactivities, and its value (11.91 %) for samples containing all the cultures was double that of the control. Liberated peptides showed high radical scavenging activities with 1,1-diphenyl-2-picrylhydrazyl and 2,20-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), (IC_{50} = 1.51 and 1.63 mg/mL, respectively) and strong antimutagenicity (26.35 %). These probiotics enhanced the generation of bioactive peptides and could possibly be commercially applied in new products or production of novel anticancer peptides.

As is evident from all stated investigations, the addition of herbal extracts to the milk and the milk fermentation by different starter cultures could ensure the production of variety novel functional yoghurts with pronounced antioxidative

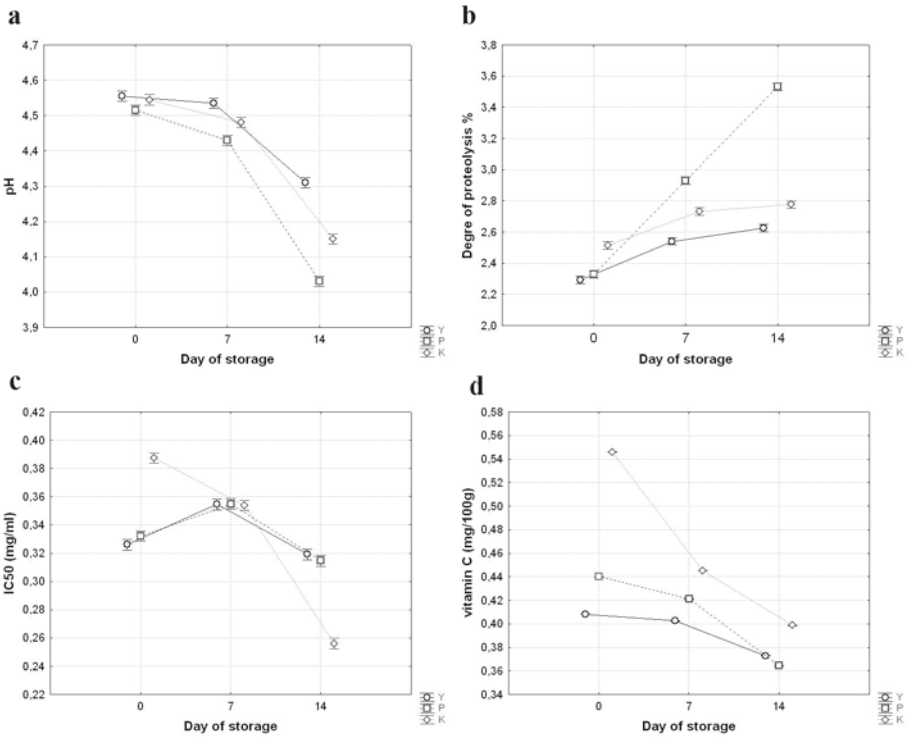

Fig. 9.3 The changes during the storage period of fermented dairy products: (**a**) pH value; (**b**) degree of proteolysis (DP); (**c**) IC_{50} values of the angiotensin converting enzyme inhibitory activity; (**d**) content of vitamin C. *Y* Yoghurt, *P* Probiotic fermented dairy product, *K* Kombucha fermented dairy product (Hrnjez, Vaštag, et al., 2014a; Hrnjez, Vukić, et al., 2014b)

activity, phenols content as well as unique sensory characteristics and extended shelf-life. Either way, enhanced functional properties expressed through antioxidative activity could not only be the consequence of the added herb phytochemicals but also to the formed bioactive peptides. Namely, supplementation with different herbs could lead to enhanced survival of starters and therefore to increasing proteolytic activity during fermentation and refrigerated storage. It has been shown that casein and whey proteins have antioxidant capacity that are responsible for the antioxidative and prooxidative activities of dairy products presumably based on their ability to bind transition metals and scavenge free radicals (Rival, Boeriu, & Wichers, 2001; Tong, Sasaki, McClements, & Decker, 2000). Some of the milk protein-derived bioactive peptides have been considered as a novel class of antioxidants (Pihlanto, 2006). Since both plain and herb-yoghurts contain casein and whey proteins, it seems obvious that phytochemicals from herbs have significant influence on its stability and degree of proteolysis and therefore they are the reason for high difference in antioxidative properties between these two products. Consequently, incorporation of novel natural flavourings and components in fermented dairy products could ensure manufacture of products with health enhancing nutraceuticals and functional properties.

9.5.2 ACE Inhibitory Activity

The therapeutic effects of fermented milks on cardiovascular diseases have been known since ancient times. They were used for high blood pressure, diabetes and heart disease with detection relatively strong ACE angiotensin-I converting inhibitory activity. The angiotensin-I converting enzyme (ACE-I) is important intermediary factor for controlling hypertension (Hernandez-Ledesma, Martin-Alvarez, & Pueyo, 2003). ACE-I convert angiotensin I to II, and both ACE-I and ACE-II are a potent vasoconstrictor and stimulator for the synthesis and release of aldosterone which subsequently increases blood pressure. ACE inhibitors reduce the activity of the renin-angiotensin-aldosterone system (RAAS) as the primary causal event in the development of hypertension. Captopril and other synthetic ACE inhibitors, used in the clinical treatment of hypertension, could have some undesirable effects to humans (Acharya, Sturrock, Riordan, & Ehlers, 2003). Therefore, natural ACE inhibitory food components have gained great importance. Food protein-derived peptides, such as casokinins and lactocinins, have shown great promise as functional food ingredients with the potential to control hypertension. ACE inhibitory activity of fermented dairy products have been mainly attributed to the peptides encrypted in major milk proteins and activated by enzymatic proteolysis, e.g. during fermentation and/or gastrointestinal digestion. The most important ACE inhibitory peptides in milk are casein-derived casokinins and whey-derivative lactocinins (Beermann & Hartung, 2013; Korhonen, 2009). Calpis is a commercial product which contains two antihypertensive peptides, Val-Pro-Pro and Ile-Pro-Pro, purified from sour milk obtained with *Lb. helveticus* and *Saccharomyces cerevisiae*. Production of these peptides is primarily dependent on the proteolytic capacity of microorganisms taking part in milk fermentation as well as process parameters, e.g. temperature. So far, in fermented dairy products, such as cheese and yoghurt, the potential antihypertensive (ACE inhibitory) peptides have been mostly studied (Gonzalez-Gonzalez, Gibson, & Jauregi, 2013; Hrnjez, Vaštag, et al., 2014; Papadimitriou et al., 2007). Besides that, the flavonoid-rich components also have the ability to inhibit ACE activity. Increased interest in the approach of inhibition ACE is reflected in the discovery of novel functional fermented dairy products with inhibitory effects (Jäkälä & Vapaatalo, 2010). Almost all investigation are focused to achieve better proteolysis using different conventional or non-conventional starter cultures, in addition to flavonoid-rich ingredients, or use of milk with different origin. The proteolitic systems of LAB consist of: (a) cell-wall proteinases, as initiators of proteolysis; (b) transport system in bacteria cell that relieve the uptake of oligopeptides and (c) system of the intracellular peptidases (Lopez-Fandino, Otte, & Van Camp, 2006). The technological challenges, thus, lie in the production of functional fermented dairy products with high concentration of specific bioactive peptides which would remain stable upon gastrointestinal digestion and/or production of protein hydrolisates with improved functionality and bioactivity.

Shori, Baba and Chuah (2013) enhanced proteolysis of milk proteins by inclusion of fish collagen and therefore improve ACE inhibitory activity and sensory

properties of the final product. Authors used fish collagen and extract of *Allium sativum* in milk fermentation in order to combine their different nutritional and functional effects. As it was observed, the presence of fish collagen could provide additional free amino acids which stimulate the growth of starter culture and its proteolytic activity leading to more extensive milk protein degradation during yoghurt fermentation (El-Ghaish et al., 2010). Thus, even small differences in protein degradation may lead to the improvement of development and formulation processes of yoghurts capable of modulating physiological effects. Accordingly to that, higher ACE-I activity of this products was observed, fresh control-yoghurt had ACE-I inhibitory activity about 40 % whereas fresh fish collagen-yoghurt and *A. sativum*-yoghurt with/without fish collagen showed almost 50 % of ACE-I inhibitory activity (Shori et al., 2013).

The higher ACE inhibitory as well as antioxidant activity was observed in fermented camel milk compared to bovine fermented milk by *Lb. rhamnosus* PTCC 1637 (Moslehishad, Ehsani, Salami, & Moosavi-Movahedi, 2013). This may be the consequence of structural differences and the higher presence of proline content in the primary composition of camel milk caseins compared to bovine milk.

Application of *Lb. helveticus* in milk fermentation was also found to increase ACE inhibitory activity. In comparison to other LAB, *Lb. helveticus* has stronger proteolytic activity and therefore provide final fermented milks with desirable physiological effects. Hugenholtz (2013) and Yamamoto, Akino and Takano (1994) also determined ACE inhibitory activity of several peptides derived from casein by an extracellular proteinase produced by *Lb. helveticus*.

Considering different microbiological composition of kombucha as an innovative starter culture in dairy industry, its application could also lead to various proteolytic degradation of milk proteins (Hrnjez, Vukić, et al., 2014; Iličić et al., 2013; Kanurić et al., 2011; Vukic et al., 2014). Hrnjez, Vaštag, et al. (2014) investigated the effects of kombucha inoculum as a new starter culture for milk fermentation on (ACE) inhibitory activity and degree of proteolysis (DP), compared to yoghurt (YF-L812) and probiotics (ABT-10) starter cultures. The authors monitored (ACE) inhibitory activity and concluded that fermented milk beverage produced by kombucha starter had the highest ACE inhibitory activity during 14 days of storage (IC_{50} = 0.25 mg/mL, Figure 9.3). This confirms that proteinase and peptidase activity of starter cultures may affect the milk protein breakdown to various extents and thus can yield a wide range of peptides with functional properties, such as ACE inhibitory activity. Even though kombucha fermented dairy products showed similar trend of changes in pH and degree of proteolysis during storage period. As some polyphenol compounds are presented in kombucha starter, it also could contribute to the ACE activity and should be further investigated. They confirmed that the kombucha starter is suitable for milk fermentation, equally good as conventional yoghurt and commercial probiotics starter cultures which makes kombucha inoculum potentially a new starter in dairy industry.

Shori and Baba (2013) produced yoghurt by LAB mixture and addition of *Azadirachta indica* water extract into pasteurized full cream milk in concentration of 10 %. Sample produced with *A. indica* water extract had 79.70 ± 11.20 % higher ACE inhibitory activity compared with control sample after 14 days of storage.

Obtained results indicated that *A. indica*-yoghurt may have the potential to serve as enhanced functional yoghurt with antihypertension activities. Amirdivani and Baba (2011) investigated possibilities of peppermint (*Mentha piperita*), dill (*Anethum graveolens*) and basil (*Ocimum basilicum*) as antihypertensive ingredients in yoghurt enrichment. Authors produced samples by additing each herbal water extract in concentration of 10 % prior to the fermentation process. All herbal yoghurts showed higher antihypertensive activity than plain yoghurt at corresponding storage periods (0, 7, 14, 21 and 28 days).

Gonzalez-Gonzalez et al. (2013) investigated novel probiotic-fermented milk with ACE inhibitory peptides produced by *Bif. bifidum* (MF 20/5). Peptide and amino acid profiles and the ACE inhibitory activity were determined and compared with those obtained in milk fermented by *Lb. helveticus* (DSM13137). The sequences of the major bioactive peptides present in fermented milk of *Bif. bifidum* and *Lb. helveticus* were identified and quantified. *Bif. bifidum* released a larger amount of peptides than *Lb. helveticus*. Also the lactotripeptide concentrations and ACE inhibitory activity were higher in *Lb. helveticus* fermented milk when the pH was maintained at 4.6. Thus, these findings show the potential for the use of this probiotic strain to produce fermented milk with a wider range of health benefits including reduction of blood pressure.

Generally, the use of non-conventional starter cultures in milk fermentation as well as the addition of functional ingredients could be considered as great possibility for the development of innovative fermented dairy products with higher ACE inhibitory and overall better biological activity.

9.6 Conclusion

Nowadays, advanced researches in manufacturing of fermented dairy products with improved nutritional and functional properties are in main focus of the dairy industry. Technological processing parameters, milk type and characteristics (cow, goat, sheep, camel), synergistic interactions between microorganisms of applied starter culture and its ability to produce exopolysaccharides as well as milk supplementation with different natural flavourings and food ingredients (dietary fibre-inulin, oligofructose; milk proteins/whey protein concentrates, transglutaminase, etc.) have been reported to influence the structural, nutritional and functional characteristics of fermented dairy products.

Novel techniques, such as high-pressure processing, high-pressure homogenization, ultrasonic processing and pulse electric field, could be successfully applied alone or in combination with other preservation methods or transglutaminase, in new fermented milk processing, particularly yoghurts with low-fat content.

The addition of natural ingredients from animal and plant origin (i.e. phytochemicals) to the milk and the milk fermentation by different starter cultures, especially probiotics and/or kombucha, could ensure the production of innovative functional fermented dairy products with pronounced antioxidative activity, ACE inhibitory and overall biological capacity of the final product.

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Chapter 10
Novel Fermented Meat Products

Derek F. Keenan

10.1 Introduction

Fermented meats are 'traditional/heritage' processed meat products whose origins traverse many ancient human civilisations (c.a. 3500 years old). They are highly diverse, differing as a result of environmental and geographic conditions that ultimately dictated their composition, i.e. from the availability of ingredients and the meat used to differences in their preparation methods (Demeyer, Toldrá, & Leroy, 2014). Meat fermentation is a preservation method that has the following major steps:

1. A low energy biological acidification process that reduces the pH through the formation of lactic acid as a by-product of carbohydrate catabolism
2. A reduction in water activity a_w from the addition of salt and drying process.

This imparts distinctive physico-chemical and sensory characteristics (Ockerman & Basu, 2016). Flavour development is further enhanced in a complex manner by specific enzymatic reactions in the meat and the microorganisms themselves, triggered by the fermentation process, which produces numerous low molecular weight flavour compounds such as peptides, free amino acids, aldehydes, organic acids and amines (Claeys, De Smet, Balcaen, Raes, & Demeyer, 2004). Traditional products would have been fermented by 'wild' microorganisms, ubiquitous in the surrounding environment. However, it wasn't until the mid-nineteenth century that the essential role of yeasts, moulds and bacteria in such products came to be fully understood. This led to the formalising of a controlled and efficient fermentation process that is the forerunner of many contemporary products we consume today (Caplice &

D.F. Keenan (✉)
Food Chemistry and Technology, Teagasc Food Research Centre Ashtown, Dublin 15, Ireland
e-mail: derek.keenan@teagasc.ie

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Fitzgerald, 1999; Ojha, Kerry, Duffy, Beresford, & Tiwari, 2015). The addition of curing salt and limited oxygen in the environment select for specific microbiota, such as lactic acid bacteria (LAB) and coagulase-negative Staphylococci (CNS). These organisms flourish due to their adaptation for the meat substrate and the aforementioned conditions (low a_w and absence of O_2). Nowadays, LAB organisms would be added in the form of a starter culture to improve standardisation of the fermentation process. The LAB initiate fermentation, lowering pH to a final range 4.5–5.5 and induce the denaturation of salt-soluble proteins to a sliceable gel, with the rate of pH decline, addition of curing salt and lowering of the water activity ultimately determining the shelf-life and safety of the products (Demeyer et al., 2014). Considerable amounts of research have been carried out on fermented meats in the past few years. Some of the major areas being explored include:

1. Scientific understanding of the products in order to produce better, more economical and consistent products. These research studies have focused on microbiota identification through genetic means in order to better improve starter culture strains and investigating the increasing importance of the role of enzymes in the development of the physical and sensory characteristics of fermented meats.
2. Improved health and safety—health is being viewed as a major driver of consumer purchasing decisions. As such, interest in not only healthy and nutritious foods has increased but also in products that may offer some additional benefits. Functional foods have been developed to satisfy this need, with functional fermented meats the subject of many recent studies. Furthermore, the safety of fermented products is a key consideration in their development. The emergence of novel non-thermal and thermal technologies could provide improved safety and stability whilst maintaining the inherent characteristics of the products and/or be used in combination with additive reduction strategies for healthier fermented meats without compromising safety.

10.2 Market and Products Consumed

The global processed meat market was reportedly worth $482 billion in 2013 and is expected to reach >$900 billion by 2020 (ReportBuyer, 2016). Due to the widespread consumption of fermented meat products, the market would seem to be considerable. However, difficulties arise in estimating the true collective value of the fermented meats market. This is due to a number of factors: (1) the diversity of the segment means it is difficult to group under one collective heading of fermented meats, i.e. numbers for production and consumption can often be collected on specific products, e.g. dry sausages; (2) owing to the historical and narrow regional nature of the some of the products, they can often be produced and consumed with no quantities being recorded (Ockerman & Basu, 2016). However, European production of fresh and fermented sausages was estimated between at 2,909,000 tonnes in 1998 (Hui et al., 2004). Table 10.1 is a mixture of traditional and commercial

Table 10.1 Selected fermented meats from different regions around the world

Region	Country/county	Example
North America	Lebanon county, PA	Lebanon bologna
	Various	Pepperoni
South and central America	Mexico, Brazil, Uruguay, etc.	Italian Milano, Cacciaturi
	Andes region	Fermented sausages (with Llama meat and guanaco)
Mediterranean	France	Menage, saucisson d'Alsace, varzi (salamis); Bayonne (dry-cured ham)
	Spain	Salchichón, chorizo (salamis); Serrano, Ibérico (dry-cured ham)
	Italian	Turista, copa, crespone (salamis); mortadella bologna; prosciutto di Parma, San Danielle (dry-cured hams)
Northern Europe	Germany	Greußner, rügenwalder
	Austria	Katwurst
	Sweden	Metwursk
	Norway	Toppen, trondermorr, stabber
	Finland	Poro meetwurst
	Iceland	Lambaspaeipylsa
Eastern Europe	Poland	Krakowscha, kabanosy, jalowcowa
	Hungary	Hungarian salami
	Romania	Hermannstädler
	Russia	Moscow salami
The middle East	Various	Dry-fermented sausages
Africa	South Africa	Biltong
	North-East Africa	Miriss, mussran
	Sudan	Beirta
East Asia	China	Lap cheong, aap gon cheong, gam ngan cheong
	Thailand	Sai ua, nham, goon chiang
	Philippines	Longamisa
	Korea	Sundae, soonday
Pacific Rim	Australia	Pepperoni

Table summarised from the work of Toldrá and Hui (2015)

produced fermented meats and while the list is not exhaustive, it attempts to give a cross section of the products available in various regions as an example of the strength and diversity of this market cohort.

10.3 Basic Composition

Fermented meats are a highly diverse segment of processed meats that are typically composed of most of the following key ingredients:

10.3.1 Meat

As is the case with all meat products, the raw meat used in the production of fermented meats should be of a high quality (Heinz & Hautzinger, 2007). In particular, it should be free of bacterial contamination as the processing takes place at temperatures ideal for pathogenic bacteria to flourish. For example, pork is susceptible to infection by a genus of parasitic roundworms called *Trichinella spiralis* (Keenan, 2016), which is typically destroyed with adequate thermal processing. However, as fermented products are often not cooked during processing or before consumption, microbial safety of raw meat is essential. Similarly, meats should be free of chemical contamination, and care should be taken where meats with higher contents of unsaturated fats (poultry) are used that may lead to rancidity issues through oxidation. Some of the common meat species used to produce fermented meats are beef, pork, lamb, chicken, duck, water buffalo, horse, donkey, reindeer, gazelle, rabbit and other animal by-products. Initial pH of the raw meat is important (typically 5.5–5.9 for most common meat types) with producers favouring a lower pH (Ockerman & Basu, 2016). Therefore, meat with quality defects such as pale soft exudate (PSE) and dark, firm, dry (DFD) are to be avoided in order to produce optimum quality products.

10.3.2 Inoculum and Starter Cultures

10.3.2.1 Inoculum

Historically, fermented meat products depended on wild inocula (natural contamination, natural inocula, back slopping, mother batch, indigenous microorganisms) obtained from the environment and/or equipment and/or previous fermented products (Ockerman & Basu, 2016). While these wild inocula conformed to no specific species of microorganisms, they were usually composed of strains of Lactobacilli and Staphylococci. They were superseded by starter cultures, which were developed to lower the pH in shorter timescales. Although controlled, industrially produced fermented meat products are often considered inferior in sensory quality to traditional artisan products fermented by indigenous microflora, the latter can be affected by poor consistency and additional safety concerns arising out of a more variable fermentation process. Therefore, starter cultures are commonly applied in contemporary fermented meat products to offset these issues.

10.3.2.2 Starter Cultures

Meat starter cultures can be defined as preparations of viable microorganisms that develop desired metabolic activity in meat (Hammes, 1990). Starter cultures are added to most contemporary fermented meat products to improve consistency and

safety. The indigenous microflora of meat usually contain low levels of LAB and CNS (10^{3-4} cfu/g), which increase during the ripening stage (10^{8-9} cfu/g). However, native levels of Enterobacteriaceae can be higher in the raw meat than LAB and CNS sp. (10^5 cfu/g) (Demeyer et al., 2014). Addition of starter cultures can, therefore, promote safety by overwhelming these potentially pathogenic strains through enhanced competition. Research into starter cultures began in the 1940s in the United States by inoculating meat batters with Lactobacilli in order to accelerate fermentation. Subsequent development in Europe in the late fifties using mixed cultures of Micrococci sp. and *Pediococcus cerevisiae* led to the development of the first generation of meat bacterial starter cultures derived mainly from vegetable fermentations. Successive new generations isolated from meat (*L. sakei* and CNS) took place in the 1990s, forming the basis of many industrial starter cultures used today (Cocconcelli & Fontana, 2010). Tailored starter cultures containing a mixture of genera and species can be designed on the basis of specific processing and product requirements. The characteristics of microorganisms considered for starter cultures along with their primary functions are presented in Table 10.2.

Commercial starter cultures are available as freeze-dried powders (most popular until recently) and concentrated hypertonic liquid suspension (Ojha et al., 2015). Starter cultures can decrease the fermentation time compared to natural fermentation by 15–20 %, improve product yield 5–7 %, and subsequently reducing ripening times (Ockerman & Basu, 2016). Starter cultures can be broadly categorised into two major categories: (1) bacterial and (2) fungal [(a) yeasts and (b) mould].

Table 10.2 Important characteristics of microorganisms considered for starter cultures and their primary functions

Characteristics	Primary functions/roles
Non-pathogenic	Acid production
Phage resistant	Catalase activity
Free of microbial/chemical impurities that inhibit safety/manufacturing	Nitrate reduction
Salt tolerant	Flavour formation
Fast growing (brine 6 %)	Bacteriocinogenic and biopreservation
Grows well in the presence of nitrite (80–100 ppm)	Novel/functional (probiotics)
Optimum growth temperatures between 26.7 and 43 °C	
Produces lactic acid	
Positively affects flavour and colour development	
Non-gas forming	
Catalase positive	
Produces little or no acetic acid	
Does not produce off-flavours during fermentation	
Phenotypically and genetically stable	

Bacterial Starter Cultures

In meat fermentation, two main microbial groups are considered important: LAB and CNS (Cocconcelli & Fontana, 2010). LAB are usually present in low amounts (3–4 log cfu/g) initially, becoming dominant during the fermentation step (Demeyer et al., 2014). The primary function of Lactobacilli is to produce lactic acid and acidify the meat. Lowering the pH denatures the proteins which in turn forms a gel, releasing moisture uniformly. *Lactobacillus* includes up to 150 different species all with varying phenotypic, physiological and biochemical traits (Axelsson, 2004), including *L. plantarum*, *L. casei*, *L. sakei* and *L. acidophilus*. Lactobacilli strains are more commonly associated with European fermented meats, while Pediococci are often characteristic of fermented meats originating from North America (Cocconcelli & Fontana, 2010).

Staphylococci sp. are more associated with flavour development with common examples being *S. xylosus* and *S. carnosus*. Some common LAB and CNS species in fermented meats are shown in Table 10.3. However, this does not reflect the true microbial diversity that may exist between species and individual product ecologies. In recent years, molecular methods (e.g. comparative genetics, microarray analysis, transcriptomics, proteomics and metabolomics) have been very helpful in identifying the true interspecies diversity present in fermented meats, and this has been comprehensively reviewed in many studies (i.e. Albano, Henriques, Correia, Hogg, & Teixeira, 2008; Cocolin, Dolci, & Rantsiou, 2011; Giammarinaro, Leroy, Chacornac, Delmas, & Talon, 2005; Leroy, Giammarinaro, Chacornac, Lebert, & Talon, 2010; Paramithiotis, Drosinos, Sofos, & Nychas, 2010). Furthermore, a recent study by Ojha et al. (2015) comprehensively reviews some of the most recent studies on the effects of starter cultures on the technological quality of fermented meats. Combinations of these microorganisms make up the majority of commercial starter cultures currently available on the market. The composition of selected starter cultures used in fermented meats is shown in Table 10.4.

Yeast Starter Cultures

To date, the role of yeast in sausage flavour formation is not fully characterised. However, it is thought that yeast cultures play an important role in lipolytic and proteolytic activities and could positively affect flavour development. The most commonly associated species associated with fermented sausages include *Debaryomyces*, *Rhodotorula*, *Hansenula* and *Candida* (Gardini et al., 2001). Of these, the *Debaryomyces* strain, *hansenii*, has been recommended as suitable for use in a starter culture preparation as it is reported to improve aroma profile (Andrade, Córdoba, Casado, Córdoba, & Rodríguez, 2010; Bolumar et al., 2006; Flores, Durá, Marco, & Toldrá, 2004) and shown to have contributed to proteolytic activity (Patrignani et al., 2007).

Table 10.3 Prevalence of LAB and CNS species in selected tradition dry-fermented products

Product	Country	Species				Reference
LAB		*L. sakei* (%)	*L. curvatus* (%)	*L. plantarum* species (%)	Other (%)	
Sausage	France	95			5 (*Enterococcus*)	Ammor et al. (2005)
	Greece	19	48	20	5 (*L. casei/paracasei*)	Rantsiou et al. (2005)
					<5 (*L. alimentarius*)	
	Hungary	71	7	<5	13 (*Weissella*)	Rantsiou et al. (2005)
	Italy	49	30	12	<5 (*Weissella*)	Urso, Comi, and Cocolin (2006)
	Argentina	55	5	40		Fontana, Sandro Cocconcelli, and Vignolo (2005)
	Spain (salchichon)		59			Aymerich et al. (2006)
Salami	Italy	60	36	<5	<5 (*Leuconstoc*)	Cocolin et al. (2009)
	Spain (chorizo)	89			*Leuconstoc mesenteroids*	Aymerich et al. (2006)
CNS		*S. xylosus* (%)	*S. sapro phyticus* (%)	*S. eqorum* (%)	Other (%)	
Sausage	France	11	12	58	8 (*S. succinus*)	Leroy et al. (2010)
	Italy	40	2	11	15 (*S. warneri*)	Iacumin, Comi, Cantoni, and Cocolin (2006)
	Argentina		100			Fontana et al. (2005)
	Spain (salchichon)	73			12 (*S. warneri*)	Martín et al. (2006)
					12 (*S. epidermidis*)	
Salami	Italy	45	17		17 (*S. lentus*)	Mauriello et al. (2004)
					14 (*S. warneri*)	
	Spain (chorizo)	81			8 (*S. warneri*)	Martín et al. (2006)
					6 (*S. epidermidis*)	

Where *LAB* lactic acid bacteria, *L. lactobacillus*, *L. plantarum* species includes *L.-plantarum*, *-para-plantarum* and *-pentosus*, *CNS* coagulase-negative Staphylococci, *S. staphylococcus*, % of isolates identified to species. Data adapted from Leroy, Lebert, and Talon (2015)

Table 10.4 Common commercial starter culture species and their primary functions

SC species	Primary functions/roles
Lactobacillus curvatus and *Staphylococcus carnosus*	Fast acidification
	Mild positive aroma development
	Stable colour
Pediococcus acidilactici and *Pediococcus pentosaceus*	Normal acidification
	Positive aroma development
	Stable red colour formation
Staphylococcus xylosus and *Pediococcus pentosaceus*	Initial fast acidification
	Medium pH decline
	Strong colour formation and stability
	Aromatic flavour development
Pediococcus acidilactici, *Lactobacillus curvatus* and *Staphylococcus xylosus*	Fast fermentation
	Distinct, good flavour
	Strong colour formation and stability
	Bacteriocin-producing sp. help control L. monocytogenase
Lactobacillus sakei and *Staphylococcus carnosus*	Mild acidification
	Positive mild flavour development
	Stable colour
Lactobacillus pentosus and *Staphylococcus carnosus*	Intermediate acidification
	Aromatic flavour development
Staphylococcus equorum	Flavour development
	Nitrate reduction

Adapted from Cocconcelli and Fontana (2010)

Mould Starter Cultures

Moulds play an important role in the ripening of many traditional Mediterranean-style-fermented meats. Research has shown that superficial inoculation of sausages with atoxigenic moulds, i.e. *Penicillium* or *Mucor* sp. contributes to sensory quality (Bruna et al., 2001; Bruna, Fernández, Hierro, Ordóntez, & de la Hoz, 2000; Garcia, Casas, Toledo, & Selgas, 2001). These sensory developments have been attributed to a number of actions carried out by the moulds, such as lactate oxidation, proteolysis, degradation of amino acids, lipolysis, lipoxidation, delay of rancidity and reduced water loss due to slower evaporation (Benito, Rodrı´guez, Martı´n, Aranda, & Córdoba, 2004; Sunesen & Stahnke, 2003; Sunesen, Trihaas, & Stahnke, 2004). Furthermore, mould coverage often confers desired visual characteristics that contribute to overall attractiveness of fermented meats which is attributed to improved colour stability (catalase activity), oxygen consumption and protection from light (Leroy, Verluyten, & De Vuyst, 2006).

10.3.3 Fat

Fat is an essential component of fermented sausages that contributes significantly to the characteristic sensory and technological quality, namely ease of chewing, enhanced juiciness, flavour and aroma development and plays a critical role during the dehydration stage of production (Ruiz & Pérez-Palacios, 2015). Pork back-fat is most commonly used in the production of fermented sausages, although the use of more unsaturated added-fat as a substitute for saturated added-fat has grown in popularity over the last number of years in order to produce more fermented meats that are perceived as healthier. Typically, between 20 and 30 % fat is added to fermented sausages although lower levels can be used. However, the sensory acceptance of fermented sausages is intrinsically linked to the level of fat present in the sausages (Mendoza, Garcı´a, Casas, & Selgas, 2001).

10.3.4 Salt

Salt is a major additive in fermented meats, although levels in the final product range widely from approximately 2 % to over 6 % in some traditional dry sausages (Varnam & Sutherland, 1995). It has three major beneficial effects to the meat:

1. Preservative action: Salt inhibits objectionable putrification and dangerous microorganisms and those which it does not inhibit are more or less unobjectionable, i.e. it promotes the growth of LAB. It achieves this by lowering the water activity. Water activity (a_w) describes the relationship between salt and water in the system. Pure water has a water activity (a_w) of 1.0 and raw meat of 0.99. Raw dry-fermented sausages can have water activity (a_w) as low as 0.92 as they are intended for long shelf life without refrigeration.
2. Solublising/extracting proteins: The extraction of myofibrillar proteins is essential for improving moisture retention and in forming the necessary bind and texture in the finished product. Myofibrillar proteins are salt-soluble and the addition of high salt concentration in rubs and brines extracts the proteins, which are essential for water binding and retention. Lowering the salt concentration can have a detrimental effect on the myofibrillar proteins extract and consequently affect the WHC and water binding properties. If the salt content is lowered, closer attention has to be paid to using high quality fresh, raw meat; adequate physical manipulation (tumbling, massaging); and very tightly controlled thermal processing (Claus, Colby, & Flick, 1994).
3. Flavour: Fermented meats are predominantly associated with an acidic and salty character, therefore highlighting the importance of salt in flavour development of fermented meat products. About 3.5–5 % salt in the product is generally considered as the present day upper limit of acceptability, depending on the product.

10.3.5 Nitrite

Nitrite salts can be used in the production of fermented meats. They are particularly useful in long shelf life products as they are powerful preservatives against many spoilage and food poisoning organisms. Typically, they act in the form of dissociated nitrous acid (HNO_2), provided the reducing conditions necessary are present. i.e. formation of NO and reduction of nitrosyl metmyoglobin (Ranken, 2000). However, their use can inhibit many starter culture organisms if not handled correctly. Furthermore, excessive consumption can be moderately toxic in humans and, therefore, the quantities permitted in foods have been restricted. Despite this, nitrite is considered very important in the manufacture of cured and fermented meats and is responsible for the following important beneficial effects:

1. Preservative and anti-botulinal effects: Nitrite serves as a vital bacteriostatic control over the outgrowth of spores from *Clostridium botulinum*. These extremely dangerous, toxin-producing bacteria can grow in anaerobic conditions such as those created in canning and vacuum-packing. Without the addition of the nitrite, the spores may produce vegetative cells that are responsible for producing the *Clostridium botulinum* toxin. The disease has a high mortality rate of 20–50 % and those that recover have health problems that take months to resolve. Heating is lethal to most cells and can destroy the toxin which is heat labile. However, the survival of spores in the meat without nitrite could lead to little or no competition from normal meat microflora allowing spores to thrive and produce vegetative cells and thus more toxins (Adams & Moss, 1997).
2. Flavour development: When meat is cured using nitrite, the resultant, desirable flavour is not the same as the flavour of uncured meat (Ramarathnam & Rubin, 1997). Cross and Ziegler (1965) observed that cured meat flavour was comprised of essentially the same constituents, generated by a combination of several reactions in addition to the suppression of lipid oxidation, irrespective of meat type. Nitrite has been shown to retard lipid oxidation and development of warmed-over flavour in cooked meat and meat products. It may inhibit the action of pro-oxidants in the muscle or stabilise the lipid component of membranes (Pearson, Love, & Shorland, 1977).
3. Antioxidant effects: Nitrite contributes to flavour stability by complexing with haem iron which reduces the risk of the iron acting as a potent catalyst in lipid oxidation. This also prevents WOF, which can result from excessive sodium chloride addition (Claus et al., 1994). If the pigment becomes decomposed, the haem portion becomes detached from the protein, the porphyrin ring is disrupted and finally the iron atom is lost from the haem structure.

10.3.6 Sugars

Simple sugars (e.g. dextrose, sucrose) are added (up to 1 %) as a fermentation substrate which can readily be utilised by all fermentation microorganisms. For example, northern European-fermented sausages are typified by their high acid sensorial profile. This is achieved by using a predominantly *lactobacillus*-based starter culture with high quantities of carbohydrate. In contrast, Mediterranean-style sausages use lower concentrations of glucose with Staphylococcus starter cultures and extended ripening period to develop a less intense acidic profile (Cocconcelli & Fontana, 2010). Therefore, the quantity of added sugar will directly influence the rate and extent of acidulation, i.e. final pH. A 1 % addition can result in a drop of one pH unit. If more complex sugars are used, they will be fermented slower, i.e. the production of organic acids is inversely proportional to the molecular weight of the sugars used as a function of the storage time (Ockerman & Basu, 2016). The build-up of organic acids becomes a limiting factor to the process as it inhibits bacterial growth. The residual unfermented sugars can help reduce the harshness of the salt, giving the fermented product a smoother flavour. In addition, Claus et al. (1994) stated that sweeteners have high water attracting properties and develop the surface colour of some products through browning reactions. In more traditional products where nitrate serves as a source of nitrite (e.g. saltpetre—common name for sodium nitrate), sugars/sweeteners provide the energy required for specific microorganisms to reduce NO_3 to NO_2.

10.3.7 Others Additives

10.3.7.1 Spices

Spices are common adjuncts in fermented products, imparting both characteristic flavor, antioxidant properties and as a fermentation aid due to their antimicrobial effects on certain strains of microorganisms (Ockerman & Basu, 2016). The extent of their effects on fermentation depends on the type, source and magnesium content of the spice. For example, pepper, mustard, garlic, allspice, nutmeg, ginger, mace and cinnamon contain magnesium which is a growth promoter for some cultures (Zaika, Zell, Palumbo, & Smith, 1978) and a growth inhibitor in *L. monocytogenes* and *S. areus* (Kang & Gung, 2000).

10.3.7.2 Polyphosphates

Their main function on addition is the improvement of water holding capacity and working synergistically with salt to extract myofibrillar proteins. Alkaline polyphosphates increase the pH moving it further away from the isoelectric point of the protein, thus increasing the water holding capacity. The outcome of this is that

products produced with the aid of polyphosphates retain more of their natural juices and stay moist despite heat processing and subsequent reheating due to increased water retention. Polyphosphates can chelate (bind) specific metal ions such as iron and copper which can act as catalysts in lipid oxidation and have a diminished role in flavour and colour stabilisation (Claus et al., 1994).

10.3.7.3 Sodium Ascorbate (or Sodium Erythorbate)

This is added to promote/improve colour formation, specifically the nitrosyl myochromagen pigment (stable pink colour if the product is cured with nitrite/nitrate) (Varnam & Sutherland, 1995). Sodium ascorbate has antioxidant properties that maintains colour and flavour of the product by chelating metals (copper, iron, zinc) that can promote oxidative rancidity.

10.3.8 Novel Ingredients

Alternative ingredients that perform similarly to their microbial starter culture counterparts are collectively called chemical acidulants (Leroy & De Vuyst, 2009). They have sometimes been used in the manufacture of salami-style products (Sebranek, 2004). These compounds shorten the ripening process by decreasing the pH without the need for microbial lactic acid formation. Rapid acidification has the potential beneficial actions of reduced spoilage and contamination risk, prolonged shelf life, improved colour stability and textural characteristics (Leroy & De Vuyst, 2009). However, some drawbacks have been reported with their use, such as, non-traditional or overly sour flavour profile (Bunčić et al., 1993), higher degree of rancidity and colour defects [due to organic acid inhibition of Gram positive, catalase positive, cocci (GCC)] (Lücke, 1998) and inhibited nitrite reduction (Campbell-Platt & Cook, 1995). In sausage products, the most common chemical acidulants applied are Glucono-delta-Lactone (GdL), citric acid and lactic acid.

10.3.8.1 Glucono-delta-Lactone (GdL)

GdL is very popular in semi- and un-dried sausage products. It's applied as a water soluble crystalline powder that hydrolyses to gluconic acid within a few hours, resulting in a steady decrease in pH (Barbut, 2006). This progressive acidification is partly the reason for its popularity—it results in a milder tasting product compared to other acidulants that instantly acidify the products. Due to its lactone structure (no free acid position) at room temperature, it can be added during sausage emulsification (Leroy & De Vuyst, 2009). The rate of pH decline is also linearly temperature dependant, i.e. under the heat generated in the smoking stage, the rate of ester hydrolysis increases and is partly converted to gluconic acid (Totosaus, Gault, &

Guerrero, 2000). It is commercially synthesised by aerobic fermentation of glucose with *Aspergillus niger* (or its enzymes) to form a mixture of gluconic acid and GdL, the latter of which is separated by crystallisation (Leroy & De Vuyst, 2009). GdL has both GRAS (generally recognised as safe) status according to the US Food and Drug administration and is permitted as a general food additive (E575) in the (CFR, 2015) CFR—Code of Federal Regulations. A study conducted by (Maijala, Eerola, Aho, & Hirn, 1993) showed that GdL addition in meat was effective in reducing biogenic amine-producing Enterococci and Coliforms.

10.3.8.2 Lactic Acid

Organic acids like citric and lactic acids have been used as chemical acidulants. Lactic acid has also been marketed as a sausage-making ingredient that inhibits *L. monocytogenes* (Leroy & De Vuyst, 2009). Its application in the product causes an instant drop in pH. However, liquid lactic acid applications have been shown to result in detrimental effects on texture, microstructure and moisture retention (Barbut, 2006). Encapsulation of liquid acids has been reported to offset some of these deleterious quality issues—by controlled/slow release during thermal processing (encapsulating material melts at 57 °C) rather than during the sausage manufacture itself (Sebranek, 2004). However, encapsulated acids would then only be applicable to products that undergo thermal treatments (such as the majority of products in the USA) (Leroy & De Vuyst, 2009). Product composition will vary depending on the product type, ingredients used, processing and fermentation conditions. However, the nutritional composition of a typical dry pork/beef salami product is given in Table 10.5 as an example of the levels one can expect in the final product.

10.4 Basic Processing

Meats can be fermented as whole meat pieces, e.g. country hams (sometimes fermented, e.g. Wiltshire cure), biltong and jerky, or in smaller comminuted pieces to form sausages, e.g. salami. Some more traditional products include the use of different animal parts in the fermentation, such as intestines, marrow, fat and sun-dried bones (Ockerman & Basu, 2016). A simplified processing flowchart of the main processing steps for a typical dry-fermented sausage is shown in Fig. 10.1. Weighed amounts of ingredients (meat and fat can be pre-minced) are chopped and blended under vacuum in a bowl chopper (or equivalent processing equipment). Batters are created by the chopping action of the blade (1000–3000 rpm) and are slowly mixed in a rotary motion by the moving bowl (10–20 rpm). In most commercial operations, this process is optimised for particle size and minimal damage to fat tissue, i.e. short processing times ≈5 min at 2 °C (Demeyer et al., 2014). Batters are stuffed under vacuum immediately after comminution into natural (cleaned intestines) and artificial (regenerated collagen, cellulose, co-extruded collagen) casings (Ranken,

Table 10.5 Example of nutritive composition of a typical dry salami (pork/beef)

Proximates	Units	Value per 100 g
Water	g	41.2
Energy	kcal	378
Protein	g	21.1
Fat (total lipid)	g	31.1
Saturated		11.4
Monounsaturated		14.7
Polyunsaturated		4.9
Carbohydrate (by difference)	g	0.7
Total dietary fibre (TDF)	g	0.0
Sugars	g	0.3

Source: USDA (2016)—Nutrient database

2000). Sausages are usually hung on racks in air conditioned chambers of high relative humidity (RH) for ripening. This consists of two stages:

1. Fermentation: This stage is associated with bacterial growth. Examples of time/temperature/RH: 62 h/20–28 °C/60–90 % RH for northern European sausage types and 100 h/5–24 °C/60–90 % RH for Mediterranean sausages
2. Drying: This stage is associated with product stability and flavour development. Temperature and humidity conditions would be similar between sausage types (e.g. 14 °C/78 % RH) but applied for different times, i.e. 2 weeks (northern) and several months (Mediterranean) (Demeyer et al., 2014).

Smoking is an optional step that imparts antimicrobial, antioxidant effects and specific flavour and colour components to fermented meats. It is usually applied after the red surface colour becomes fixed during fermentation, and it is more typically associated with northern European-style products than their Mediterranean counterparts. Sausages or whole muscle fermented meats are subjected by controlled combustion (300–600 °C) of wood (oak) to minimise polycyclic hydrocarbons (Demeyer et al., 2014).

10.5 Novel Processing Technologies

As with any meat product, particularly those that are consumed without cooking (e.g. raw fermented sausages), safety is of critical importance to consumers to ensure that they are protected from toxicological and microbiological hazards. Non-thermal processing technologies, such as high hydrostatic pressure (HHP) processing, pulsed-electric field (PEF), X-ray irradiation, pulsed ultra violet (UV) light, power ultrasound amongst others, are being considered across a wide spectrum of meat products for decontamination purposes and process optimisation. With this in mind, these technologies could be of benefit to fermented meat processing.

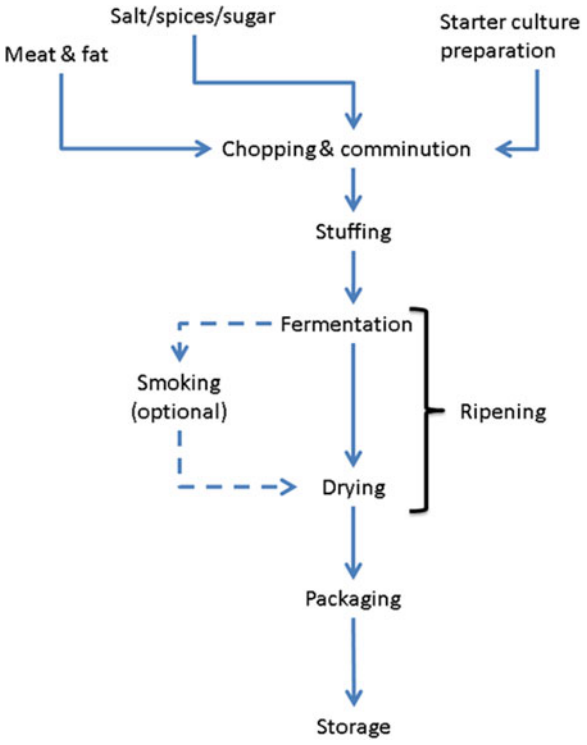

Fig. 10.1 Simplified processing flowchart of fermented sausages

High pressure treatment of foods involve the application of pressures in the range of 100–1000 MPa, and according to the isostatic principle, pressures are applied uniformly and instantaneously through a material independent of its size, shape, composition and packaging (Palou, Lopéz-Malo, Barbosa-Cánova, & Swanson, 2007). While under pressure, molecules obey the Le Chatelier–Braun principle, i.e. promoting reactions that result in a reduction of volume. Such reactions affect the structure of large molecules like proteins causing a partial unfolding of their tertiary structure. Covalent and non-covalent reactions are promoted during and after the release of pressure resulting in denaturation and thus the inactivation of microorganisms and enzymes (Hendrickx, Ludikhuyze, Van den Broeck, & Weemaes, 1998; Knorr, 1993; Oey, Van der Plancken, Van Loey, & Hendrickx, 2008). Furthermore, compounds with little secondary, tertiary and quaternary structures, such as amino acids, vitamins, pigments, flavour, aroma and bioactive compounds contributing to the sensory and nutritive quality of food, may be unaffected (Cheftel, 1992). Advantages of the technique include: It allows food to be processed at ambient or low temperature; it allows instantaneous transmittance of pressure throughout the system, independent of mass and geometry and it causes microbial destruction without resultant heat damage or use of chemical preservatives, thus improving quality (Rastogi, Raghavarao, Balasubramaniam, Niranjan, & Knorr, 2007). The majority of

HHP application in meat research is as a novel pre-/post packaging non-thermal decontamination technique in order to improve the microbiological safety and shelf life (Bajovic, Bolumar, & Heinz, 2012). In fermented sausages, many studies have shown improved product safety with the application of HHP in combination with biopreservation techniques (Garriga et al., 2005; Marcos, Aymerich, Dolors Guardia, & Garriga, 2007; Rubio, Bover-Cid, Martin, Garriga, & Aymerich, 2013). Marcos et al. (2007) reported that the application of high pressure (400 MPa/17 °C/10 min) after the ripening stage reduced *Enterobacteriaceae* without affecting sausage quality, while Rubio et al. (2013) reported that pressures of 600 MPa for 5 min at the end of ripening reduced *Enterobacteriaceae* counts to <1 log cfu/g, 1 log cfu/g reduction for *S. aureus* and *Escherichia faecium* CTC8005 and a 2 log cfu/g reduction for *L. monocytogenese*. These data show that the application of HHP during the production of low-acid-fermented sausages could lead to safer and higher quality products.

HHP application in meat products is also seen as a potential added hurdle for products in salt reduction strategies (Verma & Banerjee, 2012). Omer et al. (2010) studied the effects of HHP with reduced additives, i.e. sodium chloride and sodium nitrite on the survival of verotoxigenic *Escherichia coli* (VTEC) in Norwegian-style dry-fermented sausages. These authors concluded that HHP had the potential to make the sausages safer. Other studies have also reported on a reduction in bioactive amine generation (Garriga et al., 2005; Ruiz-Capillas & Jiménez-Colmenero, 2004; Simon-Sarkadi, Pásztor-Huszár, Dalmadi, & Kiskó, 2012). For example, Latorre-Moratalla et al. (2007) reported strong inhibition of diamine (putrescine and cadaverine) accumulation in pressurised sausage batter (200 MPa/17 °C/10 min) prior to fermentation, while Ruiz-Capillas, Jiménez Colmenero, Carrascosa, and Muñoz (2007) found significant decreases in tyramine, putrescine and cadaverine in pressurised (400 MPa/30 °C/10 min) Spanish 'chorizo' sausages.

Food irradiation as a preservation technique dates back to the 1950s, and numerous studies have been carried out in relation to its impact on meat safety and quality (Brewer, 2009). In fermented meats, Johnson, Sebranek, Olson, and Wiegand (2000) reported irradiating (1.5 and 3.0 kGy) raw materials prior to the production of pepperoni that provided a 5 log reduction of *E. coli* O157:H7 and produced a product comparable to a traditional dry sausage, while Samelis, Kakouri, Savvaidis, Riganakos, and Kontominas (2005) reported similar findings for gamma irradiated (2–4 kGy) frozen meat/fat trimmings prior to fermented sausage production. However, the latter authors also found that gamma irradiation was less promising in its control of *Listeria* spp., including *L. monocytogenes* in that study. Further work by Kim et al. (2012) reported that significant detrimental dose-dependent effects on quality (colour, lipid oxidation, sensory parameters) characteristics were observed in gamma irradiated (0.5–4 kGy) vacuum-packed dry-fermented sausages. With this in mind, and due to some other drawbacks (rise in temperature, requirement for a radiation source), electron beam irradiation techniques have shown some promise as an alternative irradiation technique. Studies by Lim, Seol, Jeon, Jo, and Lee (2008) and Cabeza, de la Hoz, Velasco, Cambero, and Ordóñez (2009) found that electron beam irradiation treatments (2 kGy) were an effective biocide in the production of food-borne pathogens, specific to dry-fermented sausage products.

Like HHP, PUVL/pulsed light (PL) have been used as a decontamination technique in food applications. PL consists of short length flashes (10^{-3}–10^{2} ms) of intense, broad spectrum light, rich in UV (Ojha et al., 2015). Decontamination occurs by way of photochemical changes to DNA caused by UV-C, photothermal and photophysical damage to cells, i.e. water vaporisation and cell membrane disruption. This makes it a very simple and cost-effective technique to increase the safety of ready-to-eat meats, like dry cured products. Ganan, Hierro, Hospital, Barroso, and Fernández (2013) evaluated the efficacy of PL in salchichón (pork dry-fermented sausage) on the inactivation of *L. monocytogenes* and *Salmonella enterica serovar Typhimurium*. Log reductions between 1.5 and 1.8 cfu/cm^2 were obtained for both organisms when an application of PL (11.9 J/cm^2) was applied.

10.6 Basic Fermentation Biochemistry

During the ripening stage, the fermented meat is a dynamic system and is affected by both microbial and endogenous enzymes (Demeyer et al., 2014). The microbial decomposition of carbohydrates, lipids and proteins are the primary factors influencing the development of characteristic appearance, flavour and texture attributes of fermented meats (Paramithiotis et al., 2010), which are shown in Fig. 10.2. During fermentation process, breakdown of macronutrients, namely carbohydrates, proteins and lipids, occurs as discussed below.

10.6.1 Carbohydrate Breakdown

Carbohydrates serve as the energy and carbon sources for the endogenous microbiota and the added starter culture. The most obvious aspect is the production of lactic and other organic acids by microbial breakdown of these carbohydrates. The formation of lactic acid isomers (D- and L-) is a function of the most dominant species of microbiota present. The result of increasing build-up of organic acids is a concomitant decrease in pH. Decreasing pH in the product promotes or initiates other fundamental changes such as an increase in product stability (inhibiting spoilage and pathogenic organism), salt solubilisation, water release as the proteins coagulate and protein hydrolysis (Demeyer et al., 2014; Paramithiotis et al., 2010).

10.6.2 Proteolysis

Initial proteolytic activity is carried out by endogenous muscle proteinases (Lücke, 2000), for example, actin and myosin are mainly hydrolysed by cathepsin D which is activated at acidic pH. The resultant polypeptides formed are further hydrolysed

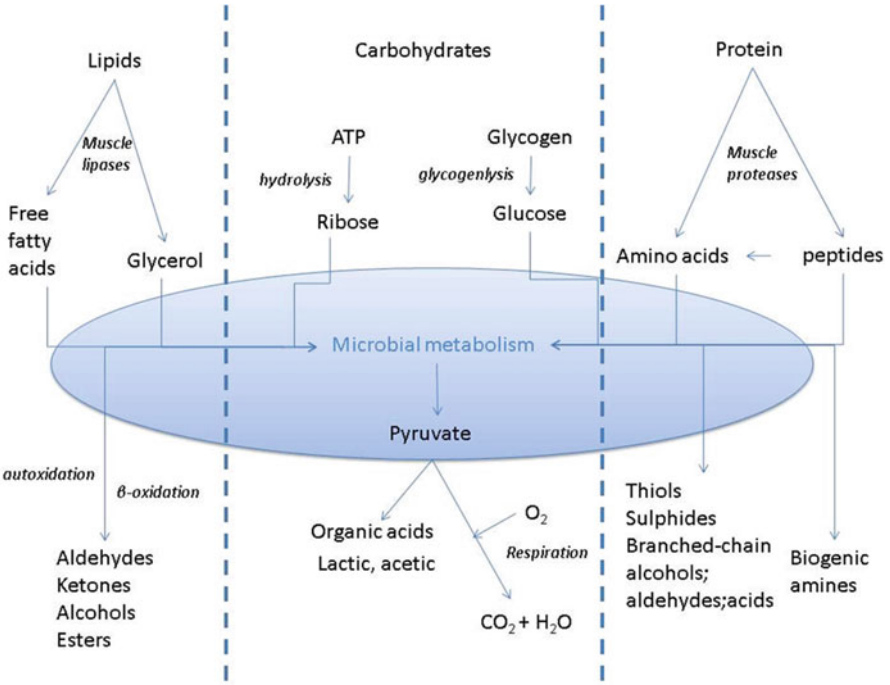

Fig. 10.2 Simplified biochemical changes during meat fermentation [based on Demeyer et al. (2014) and Paramithiotis et al. (2010)]

to smaller peptides (1–10 kDa) and amino acids by endogenous and microbial peptidylpeptidases and aminopeptidases (Demeyer et al., 2014). Amine production is generally a function of the initial contamination of the raw materials/sources within the production cycle rather than the starter cultures themselves. Interest in the formation of biogenic amines (BA) in foods like fermented meats has grown over the last few decades due to the increasing number of sensitive consumers within the general population (Cocconcelli & Fontana, 2010). They represent a health concern due to the toxicological symptoms they can exert i.e. histaminic, intoxicative and interactive behaviour with drugs (Shalaby, 1996), with excessive consumption leading to nervous, gastric, intestinal and blood pressure problems (Suzzi & Gardini, 2003). BA require the presence of amino acid precursors, microorganisms with amino acid decarboxylase activity and favourable pH and temperatures in order to accumulate (Cocconcelli & Fontana, 2010). During the ripening phase of fermentation, many of these amino acid precursors can be formed in the presence of large quantities of protein (raw material) and the proteolytic activity (Komprda et al., 2004). High levels of BA, e.g. tyramine, histamine, putrescine and cadaverine have been reported in fermented sausages (Bover-Cid, Hugas, Izquierdo-Pulido, & Vidal-Carou, 2000; Bover-Cid, Izquierdo-Pulido, & Vidal-Carou, 2000; Hernández-Jover, Izquierdo-Pulido, Veciana-Nogués, Mariné-Font, & Vidal-Carou, 1997a, 1997b).

More comprehensive reviews on the proteolytic capacity of several LAB and Staphylococci strains in fermented meats have been investigated (Drosinos, Paramithiotis, Kolovos, Tsikouras, & Metaxopoulos, 2007; Mauriello, Casaburi, & Villani, 2002; Sanz et al., 1999), which will not be discussed in detail as it exceeds the scope of this chapter.

10.6.3 Lipolysis

Lipolysis is extensively carried out by endogenous tissue lipases (between 60 and 80 %), with the rest being the result of microbial lipases (Molly et al., 1997; Molly, Demeyer, Civera, & Verplaetse, 1996). Polyunsaturated fatty acids are preferentially released due to the phospholipase activity on the muscle membrane and the specificity of fat cell lipases. Of the starter culture strains, LAB hydrolyse mono-, di- and tri-glycerides at a lower rate (Sanz, Selgas, Parejo, & Ordóñez, 1988) than their Staphylococci counterparts (Casaburi et al., 2007; Coppola, Iorizzo, Saotta, Sorrentino, & Grazia, 1997; Kenneally, Leuschner, & Arendt, 1998; Mauriello, Casaburi, Blaiotta, & Villani, 2004; Miralles, Flores, & Perez-Martinez, 1996). Once the free fatty acids are liberated, they become part of oxidative reactions forming flavour volatiles such as aliphatic hydrocarbons, aldehydes, alcohols, ketones and esters. Excessive oxidation can result in the generation of off-flavours through rancidity, which can be offset by the microbiota themselves through their consumption of oxygen (Paramithiotis et al., 2010).

10.6.4 Novel Approaches to Better Understand and Control Basic Underlying Biochemistry

The safety, shelf life and sensory characteristics of fermented meats are the result of a complex set of interacting microbiological, physical and biochemical changes that occur during ripening (Demeyer et al., 2014). Therefore, standardisation of the fermented meat process requires comprehensive knowledge of many of the interactions involved i.e. the effect of ingredients—spices (Verluyten, Leroy, & De Vuyst, 2004); non-meat proteins (Papavergou, Bloukas, & Doxastakis, 1999); the effect of processing conditions—changing temperature/RH combinations (Papadima & Bloukas, 1999); product characteristics—sausage diameter (Ruiz-Capillas & Jiménez-Colmenero, 2004). The distinctive flavour of, e.g. Mediterranean-style sausages is associated with a very specific pattern of proteolysis which is characterised by a lower peptide/free amino acid ratio as well as higher ammonia levels and dictated by specific changes in pH, dry matter, a_w,, fermentation type, fermentation temperature, length of ripening, SC used, meat type and sausage diameter (Demeyer

et al., 2014). Simple models (although often over-simplified) can be used to predict and better understand these interacting relationships:

1. Analytical models: Linear or exponential kinetics of time-related changes in metabolite concentrations, microbial characteristics or sensory quality.
2. Mechanistic models: Relate weight losses to changes in: dry matter, pH, a_w, texture; relate the kinetics of pH change to lactic acid and ammonia production, amounts of end products and substrates metabolised within the biochemical and microbial stoichiometry framework (Demeyer et al., 2014).

Other, more complicated multivariate models, such as principal component analysis (PCA) and partial least squared (PLS) regression have been found to be useful in evaluating the technological effects on the sensory and aroma characteristics of fermented meats (Casaburi et al., 2008; Henriksen & Stahnke, 1997).

10.7 Emerging and Future Novel-Fermented Products

Fermented meats are products of historical, regional and cultural significance. They are a marriage of artisan craft and industrial process that are numerous in variety, sensory characteristics and unique ecosystems for microorganisms. Standardisation of the fermentation process is reinforcing their market, and their continued development will open up new avenues to how they are viewed and consumed. Some of the future trends are listed below:

10.7.1 Functional Starter Cultures: Safety

The antimicrobial effect for ensuring the safety of fermented meats is primarily the rate acidification of the raw meat (Lücke, 2000). Despite this, other antimicrobial characteristics can be effective in reducing or eliminating pathogenic microorganisms present in fermented meat products that display acid tolerance, e.g. *L. monocytogenes* (Leroy et al., 2006).

10.7.1.1 Bacteriocin Producers

Strains that produce bacteriocins (bacteriocinogenic) are also considered desirable as they can be helpful in inhibiting several food-borne pathogens and, therefore, improve product safety. Bacteriocins are antibacterial peptides that inhibit the growth of other gram positive bacteria (Cintas, Casaus, Herranz, Nes, & Hernández, 2001; Cleveland, Montville, Nes, & Chikindas, 2001; De Vuyst & Vandamme, 1994). They are often characterised by a narrow inhibitory range that are most active against

closely related species (Eijsink et al., 2002). For example, LAB produce many bacteriocins that actively disrupt other LAB, thereby eliminating a competitor strain, but they are also known to be affective against food-borne pathogens such as *L. monocytogenes* (Leroy et al., 2006). Many bacterial strains have been screened for bacteriocinogenic properties against several food-borne pathogens, including *Lactococcus lactis* (Rodríguez et al., 1995), *L. sakei* (Aymerich, Garriga, Monfort, Nes, & Hugas, 2000) and Enterococci (De Vuyst, Foulquié Moreno, & Revets, 2003). New bacteriocin-producing strains have been isolated and used as new functional starter cultures (Coffey et al., 1998; Scannell, Schwarz, Hill, Ross, & Arendt, 2001). In situ bacteriocin production does not appear to have the disadvantages of flavour modification, which may be the case with utilising other bacterial strains. However, it is recommended that the strains are selected on their suitability for a fermented meat environment to ensure optimal performance and bacteriocin production (Leroy, Verluyten, Messens, & De Vuyst, 2002). Therefore, while bacteriocins do not represent the primary means of preservation, their appropriate integration in a multihurdle preservation protocol can help improve product safety and stability (Leroy et al., 2006).

10.7.1.2 Negative Decarboxylase Activity (Biogenic Amines)

Starter cultures with negative decarboxylase activity could prevent the growth of BA producers and lead to end products almost free of BA, provided the raw material is of good quality (Cocconcelli & Fontana, 2010). Bover-Cid, Izquierdo-Pulido, and Vidal-Carou (2001) and González-Fernández, Santos, Jaime, and Rovira (2003) reported that a selected starter culture, i.e. *L. sakei* CTC494, greatly reduced BA accumulation in fermented sausages. Furthermore, several authors have suggested that starter strains that have amine oxidase activity could further decrease the amount of BA during the fermentation process (Fadda, Vignolo, & Oliver, 2001; Gardini, Tofalo, & Suzzi, 2003; Martuscelli, Crudele, Gardini, & Suzzi, 2000; Suzzi & Gardini, 2003).

10.7.1.3 Other Antimicrobials

Antimicrobial actions, other than that of bacteriocin production, in starter cultures have also been considered. For example, the introduction of the lysostatin gene—an endopeptidase—(from *Staph. simulans* biovar *staphylolyticus*) into a Lactobacilli meat starter (Cavadini, Hertel, & Hammes, 1996, 1998; Gaier, Vogel, & Hammes, 1992) can be used to prevent the growth of *Staph. Areus* by cleaving the specific glycine–glycine interpeptide cross-bridge in its cell wall (Leroy et al., 2006). *Lactobacillus reuteri*, which produces reuterin (a broad spectrum of activity against fungi, protozoa and wide range of gram positive/negative bacteria) or reutericyclin (tetramic acid antibiotic active against gram positive bacteria), have been recommended for inclusion in starter cultures (Ganzle & Vogel, 2003; Paul Ross, Morgan,

& Hill, 2002). Antimicrobials with interesting application possibilities are continuing to be discovered and can be read in greater detail (Niku-Paavola, Laitila, Mattila-Sandholm, & Haikara, 1999; Papamanoli, Kotzekidou, Tzanetakis, & Litopoulou-Tzanetaki, 2002; Pidcock, Heard, & Henriksson, 2002; Sjogren, Magnusson, Broberg, Schnurer, & Kenne, 2003; Strom, Sjogren, Broberg, & Schnurer, 2002; Työppönen, Petäjä, & Mattila-Sandholm, 2003; Valerio, Lavermicocca, Pascale, & Visconti, 2004).

10.7.2 Functional Starter Cultures: Production and Technological Advantages

As previously discussed, bacteriocin-producing strains have the potential to improve safety-fermented meat products. Consequently, they could be effective in the control of some of the deleterious effects of food spoilage. For example, their action could be inhibitive to certain strains of LAB that produce unwanted hydrogen peroxide, product sliminess and off-odours and –flavours (Ennahar, Sonomoto, & Ishizaki, 1999). Furthermore, bacteriocin-producing strains are more competitive than their non-producing counterparts implying that their application within starter cultures may improve the overall competitiveness of the starter culture and lead to a more controlled and standardised fermentation (Vogel, Pohle, Tichaczek, & Hammes, 1993). Other functional starter cultures may be of use in reducing the levels of ingredients used in the fermentation process, e.g. nitrate and nitrite, that may have negative implications for health, i.e. nitrosamines. As nitrates/nitrites are important quality (colour) and safety hurdles (especially for the control of *C. botullinum*), it is hoped that the production of bacteriocins could compensate for this effect, while the use of strains to convert brown metmyoglobin to red myoglobin derivatives could substitute for the distinctive colour of these products (if these ingredients are reduced or removed) (Leroy et al., 2006). The latter possibility was demonstrated by Møller, Jensen, Skibsted, and Knöchel (2003) in smoked sausages using *Lactobacillus fermentum*.

10.7.3 Functional Starter Cultures: Health

Foods that have health benefits beyond those of just basic nutrition (functional foods) are increasingly being sought by more health conscious consumers. With this in mind, the meat industry is examining the possibilities of meat-based functional foods as an opportunity to improve its public image and update dietary goals (Jiménez-Colmenero, 2007). Some of these health strategies are outlined below:

10.7.3.1 Probiotics

Probiotics are live microorganisms that can confer health benefits when consumed in adequate amounts. They can be taken as a dietary supplement or consumed as part of foods, with the food acting as a carrier for the probiotic (Leroy et al., 2006). To date, the most common vehicles for probiotics have been dairy products, e.g. yogurts, but meat products, specifically fermented meats, have been considered promising candidates for their inclusion (Incze, 1998). However, meat products do not typically conform to the reputation of health foods and, therefore, this may compromise their marketing potential (Lücke, 2000). Työppönen et al. (2003) gave the most comprehensive review to date on fermented meats containing probiotics. This review focussed primarily on organisms that had the specific capabilities for meat carbohydrate fermentation. In determining the suitability of other organisms, the first step would be to determine the probiotic activity of: (1) commercially available strains (Erkkilä & Petäjä, 2000), (2) sausage isolates (Papamanoli, Tzanetakis, Litopoulou-Tzanetaki, & Kotzekidou, 2003; Pennacchia et al., 2004) and (3) intestinal isolates that perform well in the fermented meat environment (Arihara et al., 1998; Pidcock et al., 2002; Sameshima et al., 1998). Determining the effects of such microorganisms on the quality indices, particularly the sensory performance, would be an important step in determining their suitability, especially if the strains are derived from non-meat sources. Finally, clinical human intervention studies are the key to confirming efficacy, e.g. (Jahreis et al., 2002).

10.7.3.2 Nutraceutical and Micronutrient Producers

A nutraceutical is defined as substance that is considered a food or part thereof that confers medicinal or health benefits on those who consume it (Andlauer & Fürst, 2002). Most LAB have limited biosynthetic properties for the production of vitamins (Leroy et al., 2006). However, more significant amounts of vitamin production could be possible from more careful selection of strains (Lin & Young, 2000; Morishita, Tamura, Makino, & Kudo, 1999; Sybesma, Starrenburg, Tijsseling, Hoefnagel, & Hugenholtz, 2003), while metabolic engineering shows further promise to further develop cultures with in situ vitamin production capabilities (Hugenholtz et al., 2002). Conjugated linoleic acid (CLA) is a compound found mainly in the meat of ruminants that has been the focus of much food research due to its purported health promoting properties i.e. anti-atherogenic, cancer inhibition, anti-diabetic, obesity lowering and improved immunity (Belury, 2002). Certain bacterial species such as Lactobacilli, Bifidobacteria and Propionibacteria can produce CLA (Alonso, Cuesta, & Gilliland, 2003; Coakley et al., 2003; Jiang, Björck, & Fondén, 1998) and thus could be tailored as part of starter cultures to increase nutritional value of fermented meats (Leroy et al., 2006).

10.8 Conclusions

The market for fermented meats is both global and considerable in scope. However, challenges remain for them to continue as a part of our diets, most notably, the issue of health. Research has demonstrated that fermented meats have the capacity to become vehicles for health promoting compounds, such as probiotics and micronutrient-producing organisms. Furthermore, synergistic combinations of novel processing (HPP) and starter culture selection could reduce the need for such high levels of salt and nitrite, with the former acting as a microbial hurdle and the latter forming the pigments associated with fermented meats. Studies have improved our understanding of the mechanisms controlling the fermentation process in order to improving consistency, speed of production and convenience without compromising product authenticity.

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Chapter 11
Novel Fermented Marine-Based Products

Gaurav Rajauria, Samriti Sharma, Mila Emerald, and Amit K. Jaiswal

11.1 Introduction

Fermented food is currently experienced by every cultural society in the world according to the availability of the food substrate and their food consumption patterns. In many cases, such products play an important role in ethnic identity and culinary enjoyment. For instance, Europe produces the largest quantity of fermented dairy products while Africa is the largest producer of fermented starch crops and legumes based food products. Similarly, the fermented fish products are very common in south and south-east Asia whereas North America is presumably the biggest producer of fermented beverages and meat products (Khem, 2009). Over the centuries, fermentation techniques and procedure have evolved, refined and extended which helped some fermented products such as bread, cheese, butter and yoghurt to be produced all over the world.

Although, Asians were the pioneers in the development of fish-based fermentation food but marine organisms such as fish and shrimps have always been staple food for people in the coastal countries throughout the world. Because fishing is seasonal, there was a need to store and preserve fish through the winter months. Traditionally, drying and salting were the most prevalent preservation techniques

G. Rajauria (✉)
School of Agriculture and Food Science, University College Dublin, Lyons Research Farm, Celbridge, Co. Kildare, Ireland
e-mail: gaurav.rajauria@ucd.ie

S. Sharma
Institute of Medical Microbiology, Hannover Medical School, Hannover, Germany

M. Emerald
Phytoceuticals International and Novotek Global Solutions, London, ON, Canada

A.K. Jaiswal
School of Food Science and Environmental Health, College of Sciences and Health, Dublin Institute of Technology, Cathal Brugha Street, Dublin 1, Ireland

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that were in place not only to preserve food at home but also to trade food products, especially in the coastal regions (Kurlansky, 2003). However, in the case of fatty fish species (such as salmon, trout, charr and herring), the drying approach was not very effective tool for their preservations (Hagen & Vestad, 2012). In addition, the traditional preservation techniques weren't adding any nutritional value to the finished products which triggered the desire of exploring fermentation as an alternate tool for not only preservation but also for enhancing the nutritional value and flavours. Though, fermentation has always been an important part of human lives; it was not clearly understood of what actually happens during fermentation until the work of Pasteur in the latter part of the nineteenth century. Fermentation is the process by which a food can be spoiled or can be made by fermented microbes. Over the centuries, fermentation techniques have been refined and diversified for wine making, brewing, baking, preservation and dairy and non-dairy based product formation. (http://www.accessexcellence.org). However, probably the discovery of fermentation was a 'serendipity' phenomenon when friendly food microorganisms utilized the incorporated salt, and made food products more flavorous and nutritious.

Nowadays, consumer interest in fermented foods and the demand for naturally healthy and culturally embedded foods prepared with artisan technique is growing constantly. This consumer-driven trend explain the growing desire for exploring more marine-based fermented foods such as fish sauce and shrimp paste. The umami flavour, also known as the fifth taste, of these fermented products is seen as the key driver which is carving its route towards restaurants and consumer homes (http://www.prnewswire.com). Sushi and Sashimi are examples of ancient fermented Japanese fish foods that are prepared on the basis of ancient preservation methods which were the origin of such fermentation process (Skåra, Axelsson, Stefánsson, Ekstrand, & Hagen, 2015). In addition, apart from artisan taste and historically rich fermented foods, consumers are preferring foods that have beneficial components towards health and wellness. As an important aspect of this trend, probiotic fermented food are getting more attention because of their image as a gut health booster. The increased demand of traditional and/or novel value-added fermented marine products has brought new challenges to the market to develop new products to fulfil consumer demand (http://www.prnewswire.com). Therefore, this chapter focuses on various marine (animal/plant)-based fermented products currently consumed by various ethnic group worldwide. It also explores their usage through history along with current research trends and future challenges associated for their commercial production.

11.2 History of fermented food

The relation between fermented food and health dates back from Neolithic Chinese to ancient Roman era and the earliest evidence suggests that fermentation was an integral part of the old civilization. It is anticipated that it was Chinese and Georgian who prepared first fermented alcoholic beverages from fruit, rice and honey,

dates from 7000–6000 BCE. Evidence also suggests that people were fermenting beverages in Babylon, pre-Columbian Mexico and Sudan circa 3000 BC, 2000BC and 1500BC respectively (Sahrhage, 2008; Ray & Roy, 2014). The production of fermented dairy based products is well written in ancient Sanskrit and Christian scripts, while Romans were first who revealed the recipe of fermented milk preparation at around 1900 BP. In the same century, the preparation of marine based fermented foods (especially fish based) was also well practised in Europe and North Africa, but nowadays Southeast and East Asian countries are the frontrunner for its production (Khem, 2009).

Undoubtedly, Asian civilizations in particular East Asians have developed a series of fermented food products such as Lao pa daek (fish sauce) by Chinese, Mám (seafood) by Vietnamese, Natto (soybeans) by Japanese and Banchan (vegetables) by Koreans for their everyday cuisine. The other fermented foods like pickles, vinegar, sauerkraut, butter, yogurt, cheeses, and a number of fermented milk and traditional alcoholic beverages products that were developed by Asians, are still popular globally. Furthermore, fermented food such as beer and wine were also used for medicinal purposes and played an extensive role in Asians food system. Though, LAB was quiet prevalent in making traditional fermented food across the world but it was Chinese who took this research further and produced salt-fermented soyfood products such as miso, soy sauce, soy nuggets, tofu, sake, shochu (spirits), and rice vinegar (yonezu) by using fungal enzymes (Ray & Roy, 2014). The wide application of fungus in Eastern fermented foods was because of the climate of this region as fungus (especially molds) can easily grow in humid and long rainy seasons during the warm months. Contrary to East, the application of mold in food fermentation was limited in West due to their strong flavors and aromas; however, some fermented alcoholic beverages were produced by using molds (Ray & Roy, 2014).

Despite the long history of fermented food preparation and consumption, the people were unaware about the role of microorganisms, microbial enzymes and their interaction during the process of fermentation. The first breakthrough in this area came when German scientist Korschelt unveiled the role of fungus Aspergillus oryzae in the preparation of koji in 1878. The discovery of the role of another fungus Rhizopus oligosporus in fermentation fuelled the research in this area which further triggered the work on bacterial led fermentation.

11.3 Role of Microorganisms in Marine Product Fermentation

Fermented foods are foods modified by microorganisms or enzymes via desirable biochemical changes (Campbell-Platt, 1987). Though the earliest fermentation of food was carried out by resident microorganisms naturally presented on the food however, it is nowadays quite a controlled process wherein a starter culture is used to ensure a standard quality of the fermented products. Traditional fermented product is often possessing distinctive sensory qualities than the controlled process which is

believed to be due to the properties of the raw material and the traditional practices employed (Moretti et al., 2004; Ojha, Kerry, Duffy, Beresford, & Tiwari, 2015). However, such artisan fermentation can sometime deteriorate the quality and can compromise with the safety of food products. In addition, natural microflora led fermentation can also affect the uniformity of the products as the composition of so-called house-flora may vary with the manufacturing location and with the origin of raw foods. Therefore, in order to produce a more consistent and stable product with superior sensory quality and organoleptic features, a starter culture was recommended for the controlled fermentation process (Leroy, Verluyten, & De Vuyst, 2006; Ojha et al., 2015). The most auspicious microorganisms for starter culture are those isolated from native microflora of traditional fermented products. Because these microorganisms are well adapted to the selected food environment and offer a strong competition to the harmful undesirable microorganisms. The starter culture contains a mix of various type of microorganism wherein the selection of each microorganism is based on specific and required function as the functional characteristics of different bacterial strain even within the same species are always unique (Soccol et al., 2010; Ojha et al., 2015). Commercially, this careful selection of wild microorganism from traditional food can also enhance the technological innovations for novel fermented products (Leroy et al., 2006; Kołożyn-Krajewska & Dolatowski, 2012. Apart from functional specificity, the selected strain should also contain bacteriocin production capability which is a key characteristic of a microorganism to act as a starter culture. Bacteriocins, cationic peptides with hydrophobic or amphilitic properties, are mainly produced by Lactobacillus acidophilus. They are divided into the following main classes: Lantibiotics which are small peptides, and small head proteins subclasses IIa (pediocin-like bacteriocins) and IIb (two peptite bacteriocins), and helveticin (Dobson, Sanozky-Dawes & Klaenhammer, 2007). Bacteriocins demonstrate significant inhibition of a number of spoilage and food-borne pathogens, including but not limited to Staphylococcus aureus, Enterococcus faecalis, Clostridium botulinum, and Listeria monocytogenes. They have a wide antibacterial spectrum with potential applications in fermented products and foods, such as meat and fish products, fruits and vegetables, cereals and beverages (Gürakan, 2007).

Yeast, mainly Saccharomyces cerevisiae, has a long history to ferment glucose while numerous other fungi or bread moulds are regularly used to produce various value added products like alcohol, enzymes, and sugars after aerobic and anaerobic fermentation. However, of all the genus of bacteria researched, Lactobacillus, staphylococcus and Bifidobacteria have received much attention in food research. According to the Inventory of MFC, 195 species of bacteria and 69 species of yeasts and moulds have been used in the food fermentation. Among them, the bacterial genus Lactobacillus (84 species), Staphylococcus (15 species), Weissella (9 species), Acetobacter (9 species), Gluconacetobacter (9 species) and Bifidobacterium (8 species) whereas fungal genus Candida (10 species) and Penicillium (7 species) provide the largest number of species for various food matrices fermentation (Bourdichon et al., 2012). In marine product fermentation, the most popular and widely used bacterial strains are represented by the genus *Lactobacillus*. They are Gram-positive, rods or coccus-shaped, non-flagellated, non-spore-forming,

aerotolerant or anaerobic bacteria. During fermentation, lactobacilli utilize carbon sources (such as glucose) and convert it into lactic acid, carbon dioxide and ethanol (and/or acetic acid) as by-products (Hammes & Vogel, 1995; Soccol et al., 2010). LAB-led fermentation inhibits the growth of spoilage bacteria and pathogens with the help of lactic acid which consequently extended the shelf-life and the safety of products (Hu, Xia, & Ge, 2008). These bacteria are natural resident and distributed throughout the gastrointestinal tract (GIT) of human and other higher animals and are rarely associated with any kind of GIT infection. Because of their friendly nature these microorganisms have the reputation of improving gut health (Soccol et al., 2010). The effects of *Lactobacillus* on quality attributes have been extensively studied in different fermented marine products. Lactic acid bacteria are found as the dominant microorganisms in many fermented fish products. The bacteria utilize carbohydrate and reduce the pH of fermented products by producing organic acids. The reduction in pH increases the texture firmness and mouthfeel and adds a unique lactic acid flavour to the fermented product (Gelman, Drabkin, and Glatman 2000). The other genus which is quite prevalent in marine product fermentation is *Staphylococcus* which is Gram-positive, round shape, grape-like clusters and is responsible to enhance the colour and flavour of the fermented products. The lipolytic and proteolytic characteristics of few staphylococci (for instance *Staphylococcus xylosus*) contribute to the aroma of fermented sausages by producing of esters and other aromatic compounds from amino acids, whereas catalase activity of such species prevents rancidity of the products. The esterase activity of *S. xylosus* is important antimicrobial as well as crucial for the proper fermented sausage aroma (Barriere et al., 2001; Mauriello, Casaburi, Blaiotta, & Villani, 2004). Thereby mixing culture in combination with LAB and Staphylococcus contribute to the control of microbial safety and quality of the products. In this fermentation, LAB produces lactic acid and kills the harmful bacteria thus ensures the microbial safety while staphylococcus influence other technological properties through nitrate reductase and flavour forming activities (Hu et al., 2008). Talon, Walter, Chartier, Barriere, and Montel (1999) suggest that it is staphylococcus which contributes in the aroma and flavour of fermented sausage products rather than LAB. However, low pH and organic acids are the main factors that help to preserve the fermented marine food. Additionally, high salt and spices (such as garlic, pepper or ginger) may also add to the safety and quality of fermented products (Paludan-Müller, Madsen, Sophanodora, Gram, & Møller, 2002).

11.4 Current Research Trends in Fermented Marine Products

The current trends of fermented marine products are driven firstly by food authority's recommendations and secondly by the market demands. Furthermore, today's consumers are more inclined towards health and well-being and prefer mild taste and low salt. Because of these, the production and the intake of traditional fermented food are quite low as they are both salty as well as strong in taste. However,

the consumption of these type of foods among the adults (age >40 years) in northern Europe has slightly increased in recent years. The potential reason for this could be related to the scientific documents, awareness and health benefits associated with these products (Skåra et al., 2015). Furthermore, the efficient and rational use of microbes in the production of fermented food has opened up a new perspective and opportunities in this area. Advance scientific research has demonstrated the application of novel microbial cultures or revealed the greater role of our own microbiota which can not only enhance the nutritive value of fermented marine products but can also deliver various health benefits to the consumers (Bourdichon et al., 2012; Ojha et al., 2015). In addition, the application of industrial starter culture and genetically modified microorganisms can offer additional characteristics which can further enhance the functional, nutritional and health properties of the final product (Ojha et al., 2015). However, despite the major breakthrough in microbial research, the role of numerous species isolated from traditional fermented foods is still undefined (Bourdichon et al., 2012).

11.5 Marine Animal/Organism-Based Fermented Products

11.5.1 Fish-Based Fermented Products

Despite the increased dependency on fish and fish-based products across the globe, several challenges like seasonal availability and easy susceptibility to contamination have resulted in the development of certain processing technologies to deal with them. Fish and fish-based products have been preserved for ages through the civilizations. One of the major preservation methods involved the use of salts. However, due to decreased imports across the countries, the use of salt remained restricted. Of all the preservation measures of freezing, canning, etc. fermentation was the most widely used method due to the distinctive and unique sensorial properties such as flavour, colour, texture, etc. Also, it is the cheapest of all the preservation methods.

Fermented fish products are highly popular in the Indian subcontinent, parts of Africa and Europe. Fermentation protects the outer surface of fish from microbial contamination and renders the enzymes resent in the fish allowing fermented product to remain stable for a considerable amount of time (Clucas, 1982). It further breaks down the wet proteins present at the surface of the fish into substances that are simple and stable at room temperature. Depending upon the degree of this breakdown, salt is added in varying amounts to obtain the desired taste. Mainly, three different types of products are obtained using fermentation. These are (a) products in which whole or large pieces of fish are retained, (b) products where the fish is reduced to form a fine paste and (c) fish sauces where the flesh is reduced to a sauce. However, these types of products are mainly found in the Asia (Clucas, 1982). These fermented products are sold as whole fish or cut in pieces (Essuman, 1992). Following are some of the major fish-based fermented food products available in different parts of the world, most of which are confined to their specific regions are mainly known by their local names.

Herring (*Clupea harengus*) is a small salt water fish found in the North Atlantic Ocean, the North Sea and part of the Pacific Ocean. Owing to its availability, herring has been an essential part of the traditional diets in European countries like Norway, Sweden, Finland and Denmark. It is an oily fish with several essential nutrients and fatty acids. The fish is normally eaten salty. The fish is eaten as salted whole herring, herring fillets, spice-salted herring, etc. in different Nordic countries.

Surströmming is a fermented fish product staple of traditional Swedish cuisine since sixteenth century. It is made from herring caught from the northern region of the Baltic Sea thus locally known as the Baltic Herring. The fish is mainly caught in the months of May, June and early July when the fat content is low (Skåra et al., 2015). The fish is first pre-salted in a strong brine of saturated salt solution for 1–2 days with continuous stirring for the first 4 h to drain out all the blood. The fish is then beheaded and gutted and put in barrels containing 17 % salt solution and stored at 15–18 °C for 3–4 weeks. The barrels are never filled to the top and some space is always left because the fermentation process releases certain gases over the storage period. The fermented product is then transferred to cans along with the brine. However, the fermentation process continues inside the cans and upon opening these gases escape out giving a characteristic smell.

Rakfisk is another fermented fish produced from salmonid freshwater fishes like trout and char. The fermented fish is a native food of Norway produced by salting and fermenting for 2–12 months. The gutted fish is rinsed and put in wooden barrels and covered with salt or preformed brine of salt concentration 4–6 % w/w (Skåra et al., 2015). The lid of the barrels is placed in a way to provide pressure to the fish. The fish is stored in these barrels at 4–8 °C for 3–12 months.

Hákarl is a Greenland shark fermented product famous as the national dish of Iceland. These Greenland sharks are poisonous when consumed fresh due to the presence of high quantities of urea and trimethylamine oxide (Skåra et al., 2015). They are therefore processed to remove these toxic compounds. Owing to the poor production of salt in the country, fermentation and drying were the major traditional food preservation methods. Greenland sharks were cut into chunks and washed with seawater, followed by burying them into the pits prepared mainly close to the sea. This was done to make the seawater available to the pits at the time of high tides. These pits were then covered with sand and stones to compress the fish to extract all the fluids out of them. The sharks were left to ferment for 6–12 weeks depending upon the season of the year. The fermented sharks were the taken out of the pits and cut into small pieces and then dried in the air for several weeks. It is this process of fermenting and drying, which makes this shark safe and edible.

In African continent, fermentation is one of the main food processing techniques due to its ease of use, cost efficient and ability to increase the storage of meat products and reduction in spoilage over considerable time period. Of all the fermented products, fish-based fermented products are increasingly popular across the continent and one of the main source of animal protein in the diet. These fermented fish products are produced in three main forms as (a) fermentation with salting and drying, (b) fermentation with drying without salting and (c) fermentation with drying without salting (Anihouvi, Kindossi, & Hounhouigan, 2012).

Based on these three basic approaches of fermentation, some of the most famous fermented fish products in Africa are *Lanhouin*, *Momone* and *Guedj* (Anihouvi et al., 2012). *Lanhouin* is fermented fish product widely used in urban rural areas in Southern Benin, Togo and Ghana. It is mainly prepared from cassava fish (*Pseudotolithus* sp.) or Spanish mackerel (*Scomberomorus tritor*) by spontaneous and uncontrolled fermentation that consists of dressing of fresh fish followed by 10–15 h of ripening (Anihouvi, Ayernor, Hounhouigan, & Sakyi-Dawson, 2006). Later, the fish is salted and allowed to ferment. The fish is wrapped properly and buried in 2 m deep pit and left to ferment for 3–8 days. The fermented fish is then washed to remove excess salt and sundried for 2–4 days.

Momoni is prepared from fresh water fish African Jack Mackerel (*Caranx hippos*) by scaling, gutting and washing with tap water. The fish is then salted with 294–310 g/kg. The gill and gut region is highly salted and the kept in baskets covered with aluminium trays and allowed to ferment for 1–5 days. The fermented product is then washed with brine and cut in pieces, salted and sun-dried for few hours (Sanni, Asiedu, & Ayernor, 2002).

The fermented fish products found in Asia differ from that of the rest of the world. Fish sauce is a fermented brown liquid seasoning known by different local names in different parts of Asia. Some of the well-known examples include *shottsuru* in Japan, *budu* in Malaysia, *patis* in Philippines, *nuoc-mam* in Vietnam, *yu-lu* in China, *nam-pla* in Thailand and *bakasang* in Indonesia (Kilinc, Cakli, Tolasa, & Dincer, 2006). Fish sauces are mainly salt-soluble proteins prepared with the help of halophilic bacteria (Lopetcharat, Choi, Park, & Daeschel, 2001). These sauces are known for their characteristic taste and nutritive value. With about 20 g/L of nitrogen fish sauces form a major part of the protein diet in various regions in Southeast Asia. All these and many more fish-based products are specific to the regions and thus the production method might vary depending upon the traditions of that region. Owing to this wide diversity across the continent and their individual geographical needs, the Asian food market is flooded with different types of fermented fish products ranging from whole fish, sauces, pastes to products containing mixture of fish, salt, rice, spices, etc. For instance, Thai fermented fish product *pla-som* consists of fresh-water fish, salt, boiled rice and garlic (Paludan-Müller et al., 2002).

11.5.2 Other Marine Animal-Based Fermented Products

Shrimps are other marine organisms that are extremely popular in Asian countries where they are consumed as fermented paste. *Kapi*, a traditional Thai fermented shrimp paste prepared from small shrimp (*Acetes vulgaris*) or krill (*Mesopodopsis orientalis*) with solar salt in the ratio 5:1 is widely used as a condiment across the Southeast Asia (Faithong & Benjakul, 2014). The sun-dried and grounded mixture is allowed to ferment in an earthen jar for 3–6 months. Based upon the source of the raw material, Kapi is classified as *Kapi Ta Dam* (Black paste) and *Kapi Ta Deang* (red paste) obtained from mangrove canals and seagrass beds. *Kapi Ta Dam* is

mainly prepared from *M. orientalis*, whereas *Kapi Ta Deang* is produced from *Acetes indicus, Acetes japonicus* and *Acetes erythraeus*. Both these products contain high salt concentration of about 13–17 g/100 g sample (Kleekayai et al., 2015). Besides their nutritive importance, these products are also very well known for their antimicrobial, antioxidative and ACE inhibitory properties (Kleekayai et al., 2015; Peralta et al., 2008).

Oyster sauce is another marine-fermented product that is highly popular in Korea. Oyster sauce is prepared from fresh oyster (*Crassostrea gigas*) by mixing with approximately 25% (w/w) salt and fermented at 25 °C for about 6 months. These sauce have been found to contain free amino acids like glutamic acid, glycine, lysine and alanine (Je, Park, Jung, & Kim, 2005). Fermented oyster sauce is not only known for their nutritional and taste attributes but they also contain ACE inhibitory peptides that offer functional properties to the product (Je, Park, Jung, Park, & Kim, 2005). Table 11.1 lists some of the most common fermented fish and other marine products along with the region where they are mainly consumed.

11.5.3 Associated Changes After Fermentation of Marine Animals/Organisms

Preservation of fish products mainly involves the use of salt. However, the amount of salt used varies depending upon their availability, region of use and duration of fermentation. In certain cases, it is accompanied by other food processing methods of drying depending upon the kind of flavour and aroma required. Addition of salt provide the initial dehydration of the product to extract all the poisonous compounds, regulates the moisture content and also reduces the chances of contamination due to its intrinsic antibacterial nature (Beddows, 1997). Besides, salt also helps activate certain intrinsic enzymes present in the fish flesh or in the microorganisms that aid in the fermentation of the fish products. The duration of the fermentation process defines the aroma, taste and type of the final product.

11.5.3.1 Microbial Changes

Studies have identified microorganisms ranging from aerobic, anaerobic, microaerophiles, thermophiles, halophiles, etc. involved in the fermentation of fish-based products. However, the amount of these microorganisms might vary depending upon the type of fish, concentration of salt and stage of fermentation (Table 11.1). Bacterial count of 10^8 cells/g is achieved at the end of fermentation in *Hákarl* (a type of fermented shark as discussed in the previous section). Some of the predominant bacterial species include those from *Acinetobacter* group along with *Lactobacillus*. The urease enzyme present in these bacteria converts urea present in *Hákarl* into ammonia, resulting in an increase in pH from 6 to 9 in the final product (Skåra et al., 2015). During this process, TMA is also formed from trimethylamine

Table 11.1 List of fermented marine animal-based products and microorganism involved in the fermentation

Fermented products	Region	Microorganisms	Raw material	References
Surströmming	Sweden	*Halanaerobium*	Freshly caught Baltic herring (*Clupea harengus* var. membras), salt	Skåra et al. (2015)
Rakfisk	Norway	*Lactobacilli* (*Lactobacillus sakei*)	Salmonid freshwater fish, salt	Skåra et al. (2015)
Hákarl	Iceland	*Moraxella/Acinetobacter, Lactobacillus* sp.	Greenland Shark (*Somniosus microcephalus*), salt	Skåra et al. (2015)
Lanhouin	Africa (Benin, Togo, Ghana)	*Bacillus* (*B. subtilis, B. licheniformis*), *Staphylococcus* (*S. lentus, S. xylosus*), *Corynebacterium, Pseudomonas, Micrococcus* (*M. luteus*), *Streptococcus, Achromobacter, Alcaligenes*	Cassava fish (*Pseudotolithus* sp.), Spanish mackerel (*Scomberomorus tritor*), salt	Anihouvi et al. (2006) and Anihouvi, Sakyi-Dawson, Ayernor, & Hounhouigan (2007)
Momone	Ghana	*Micrococcus, Staphylococcus aureus, Staphylococcus* sp., *Bacillus* sp., *Lactobacillus* sp., *Pseudomonas, Pediococcus, Klebsiella, Debaryomyces, Hansenula, Aspergillus.*	African jack mackerel (*Caranx hippos*), salt	Nerquaye-Tetteh, Eyeson, and Tete-Marmon (1978) & Sanni et al. (2002)
Pla-som	Thailand	*Pediococcus pentosaceus, Lactobacillus alimentarius/farciminis, Weissella confuse, L. planetarium, Lactococcus garvieae, Zygosaccharomyces rouxii*	Snakehead fish, salt, palm syrup, roasted rice	Paludan-Müller et al. (2002)
Ngari	India	*Lactococcus plantarum*	Fish (*Puntius sophore*) and salt	Thapa, Pal, & Tamang (2004)
Hentak	India	*Lactobacillus fructosus, Lactobacillus amylophilus, Enterococcus faecium*	Mixture of sundried fish (*Esomus danricus*) powder and petioles of aroid plants (*Alocasia macrorrhiza*)	Thapa et al. (2004)
Tungtap	India	*Lactobacillus coryniformis subsp. Torquens, Lactococcus lactis subsp. Cremoris, Lactobacillus fructosus*	Dry fish (*Danio* spp.) and salt	Thapa et al. (2004)
Budu	Malaysia	*Micrococcus luteus, Staphylococcus arlettae*	Raw anchovies (*Stolephorus* spp.) and salt	Sim, Chye, & Anton (2015)
Yu lu	Southern and Eastern parts of China	Halotolerant and halophile groups (LAB and Yeast)	Anchovies (*Engraulis japonicus*) and snakehead fish (*Channa asiatica*)	Jiang, Zeng, Zhu, and Zhang (2007) & Jiang, Zeng, and Zhu (2011)
Kapi	Thailand, Cambodia	*Staphylococcus, Tetragenococcus*	Shrimp (*Acetes vulgaris*) or krill (*Mesopodopsis orientalis*) and salt	Chuon et al. (2014) and Faithong & Benjakul (2014)
Prahok	Cambodia	*Staphylococcus, Tetragenococcus, Rhodotorula, Candida*	Freshwater fish (*Channa striata*) and salt	Chuon et al. (2014)

Toeuk trey	Cambodia	*Bacillus, Virgibacillus, Lentibacillus, Lysinibacillus, Staphylococcus, Micrococcus, Kocuria*	Small freshwater fish	Chuon et al. (2014)
Bakasang	Indonesia	*Micrococcus, Streptococcus, Pediococcus* sp.	Small fish like sardines (*Engraulis japonicus*) and salt	Ijong & Ohta (1996)
Som-fug	Thailand, Southeast Asia	LAB	Fish *(Priacanthus tayenus, Nemipterus japonicus, Sphyraena langsar, Sphyraena obtusata obtusata, Saurida tumbil, Trichiurus lepturus)*	Riebroy et al. (2007a, 2007b)
Sausage	Asia (specially in China)	LAB (*Lactobacillus plantarum, Staphylococcus xylosus*	Fish (*Macruronus novaezealandiae, Arripis trutta, Pseudocaranx dentex, Hypophthalmichthys molitrix*)	Hu et al. (2008) & Khem, Young, Robertson, & Brooks (2013)
		Pediococcus pentosaceus		
		Lactobacillus casei subs casei)		
Miso	Japan	*Aspergillus oryzae*	Fish meat paste *(Scomber australasicus* and *Trachurus japonicus)*	Giri, Osako, & Ohshima (2010)
Pla-ra	Thailand	*Pediococcus sp.*	Freshwater fish	Lee (1997)
Sikhae	Korea	*L. mesenteroides, L. plantarum*	Seawater fish	Lee (1997)
Narezushi	Japan	*L. mesenteroides, L. plantarum*	Seawater fish	Lee (1997)
Burong-isda	Philippines	*L. brevis, Streptococcus sp.*	Freshwater fish	Lee (1997)
Gravlaks	Northern Europe	*Pediococcus, Lactobacillus, Leuconostoc, Micrococcus* and *Staphylococcus* sp.	Salmon (*Salmo salar*)	Nordvi, Egelandsdal, Langsrud, Ofstad, & Slinde (2007)
Rakørret			Trout (*Salmo trutta*)	
Tidbits and *Surströmming*			Atlantic herring (*Clupea harengus)*	
Jeotgal	Korean	*Staphylococcus equorum, Halanaerobium saccharolyticum, Salimicrobium luteum, Halomonas jeotgali*	Shrimp *(Acetes japonicus)*	Han et al. (2014)
Terasi	Indonesia	LAB *(Tetragenococcus halophilus group Tetragenococcus muriaticus)*	Shrimp paste	Kobayashi et al. (2003)
Balao-balao	Philippines	*L. mesenteroides, P. cerevisiae, S. faecalis*	Shrimp (*Penaeus indicus*) and salt	Sanchez (2008)
Alamang	Philippines	Yeast and LAB	Small shrimp	Melchor (2008)
Burong Talangka	Philippines	LAB	Live shore crabs (*Varuna litterata*)	Sanchez (2008)

N-oxide. However, the bacterial count decreases during the process of drying resulting in poor or no ammonia and TMA quantities. According to Skåra et al. (2015), several studies over the years confirm *Lactobacillus* as the dominant microbial species in the *Rakfisk*, another fermented product of Europe. The bacteria are found in lower quantities in the raw material followed by increase in their number to 10^8–10^9 cells/mL at the end of 4-weeks. *Lactobacillus* also dominates another fermented fish product called *Surströmming*. However, further ripening in the packed-canned product is mediated by a strict anaerobic halophile called *Halanaerobium* providing a unique odour to the final product (Skåra et al., 2015).

A variety of microorganisms including range of Gram-positive and Gram-negative bacteria have been identified in various African fermented fish products. Some of the most prominent species found in *Lanhouin* include *Bacillus* sp., *Staphylococcus* sp., *Micrococcus* sp., *Streptococcus* sp. and *Corynebacterium* sp. That might have come from the salt used during the treatment of the fish which favour the growth of salt tolerant microorganisms and inhibiting the growth of lactic acid bacteria. Because, in the processing of *Lanhouin*, dressing of the fish is done prior to ripening in order to minimize the chances of any spoilage due to the presence of microorganisms of the gut. In addition to some of these species, *Momone* also contains populations like *Klebsiella* and moulds like *Aspergillus*, whereas *Guedj* contains *Proteus* sp., *Shewanella putrefaciens* and *Bacillus* sp. as the predominant microbial populations. *Momone* is prepared in method similar to *Lanhouin*.

Unlike African and European fermented fish products, major products in Asia mainly comprise of whole fish, fish sauce and fish paste. Studies indicate a significant increase in the bacterial count in fish sauces in the first 8 days of fermentation that decreases thereafter. It is also seen that the fish sauces containing spices show a comparative decrease in the microbial count as compared to those that lack spices (Kilinc et al., 2006). In case of fish pastes, like *bagoong* which is native of Philippines, studies indicate that aerobic microorganisms predominate during the beginning of the fermentation process which is replaced by microaerophiles and anaerobic microorganisms. However, in certain cases, harmful microorganisms could also persist as part of the microflora. *Pla-som* a fermented fish product of Thailand contains Lactic acid bacteria isolates of *Pediococcus pentosaceus*, *Lactobacillus alimentarius*, *Weissella confusa*, *Lactobacillus plantarum* and *Lactococcus garvieae* and yeasts like *Zygosaccharomyces rouxii* as the dominant species. A study indicates an increase in number of these microorganisms within the first week of fermentation with numbers varying depending upon the salt concentration used (Paludan-Müller et al., 2002). Yeast is mainly responsible for the flavouring of this product. However, in another Thai product *som-fak*, growth of yeast indicates spoilage.

11.5.3.2 Biochemical Changes

Fermentation of the fish causes the breakdown of the fish protein by both the intrinsic enzymes of the fish and enzymes of the halophilic and halotolerant and microorganisms resulting in the formation of simple proteins, peptides, free amino acids

and ammonia. However, depending upon the amount of salt added to a product and duration of fermentation, the amount and types of these compounds present in the product varies from region to region. Some of the compounds produced by such fermentation include alcohols, acids, aldehydes, ketones, nitrogenous compounds and aromatic compounds. One of group of these compounds is responsible for distinct taste and flavour in different types of fermented fish products.

Alcohols are one of the main products of any fermentation process. Ethanol is produced as a result of the microbial fermentation of sugars present in the fish. 1-pentanol, 2-methyl 1-butanol, 2-methyl 1-propanol and 3-methyl 1-butanol are some of the alcohols present in the fermented fish products found to be responsible for their aroma. 3-methylbutanal, 2-methylbutanal and 2-methylpropanal are some of the aldehydes produced as a result of oxidation of lipids or deamination of amino acids present in the fish products over the period of fermentation. These aldehydes are responsible for characteristics like fishy and grassy; nutty and pungent smell is different fermented fish products. *Budu* is one such fish sauce that contains high concentration of aldehydes. Ketones like 2-ethylfuran and 3-pentylfuran, on the other hand, provide a cheesy odour to the fish sauces (Mohamed, Man, Mustafa, & Manap, 2012). A study by Fukami et al. (2002) has identified 2-methylpropanal, 2-methybutanal, 2-pentanone, 2-ethylpyridine, dimethyltrisulphide, 3-(methylthio)-propanal and 3-methylbutonic acid as principal contributors to odours of fishy note, sweaty note, faecal note, cheesy note, rancid note, burnt note and meaty note in fish sauce (Fukami et al., 2002).

Besides, nitrogenous compounds like 2,6-dimethylpyrazine and aromatic compounds like benzaldehyde and benzeneacetaldehyde are some the most common compounds that contribute to the aroma of fish sauces (Mohamed et al., 2012). During the fermentation process, the protein content increases and carbohydrate content decreases. Oyster sauces have been found to contain higher amounts of amino acids like aspartic acid, lysine, glutamic acid, glycine and alanine than other free amino acids. These amino acids have been found to be essential for taste in oyster sauces.

11.5.3.3 Other Changes

The microbiological changes contribute to a specific microbial cell population that along with the intrinsic enzymes of the fish help bring out biochemical changes resulting in the production of wide range of compounds that ultimately help achieve specific characteristics in the final product. Besides, the microbial population and the salt concentration help produce changes in the pH, moisture content, aroma, colour and texture.

Changes in pH occur during the course of fermentation due to the releases of different compounds like organic acids, ammonia, free amino acids, etc. *Hákarl* fermentation results in an increase in pH from 6 to 9 due to the conversion of urea present in the fish to ammonia over the fermentation process. In certain cases, the concentration of salt used and the temperature at which the fermentation occurs regulates the changes in pH of the product. For instance, in *rakfisk,* fermentation is mainly

by autocatalytic process at low temperatures whereas temperatures of 5–10 °C, it is mediated by microorganisms like lactic acid producing bacteria. Therefore, at temperatures of around 5–10 °C and 5–6% salt concentrations, pH initially drops from 6.5 to 4.5 and rises at the later stage (Skåra et al., 2015). Studies on similar fermented fish products indicate changes in the moisture content and viscosity of the final products that indicate differences in the raw materials and different concentrations of salt used for curing (Harikedua, Wijaya, & Adawiyah, 2012).

Fermentation also provides a specific colour to the final product. This is due to the release of carotenoids by the autocatalytic process. It is found that prolonged fermentation causes the release of pigment from the red–orange-coloured protein–pigment complexes (Astaxanthin) in shrimp resulting in increased redness of *Kapi* (Faithong & Benjakul, 2014). Several studies have identified sensory attributes related to taste and odour that also help differentiate the fermented fish products. Some of these attributes are sulphury meaty odour associated with garlic and meat, overripe cheese, acid vinegar, burnt odour associated to overheated product, salty taste, Umami taste associated with monosodium glutamate and sour and bitter (Harikedua et al., 2012). These sensory attributes are supposed to be related to specific physiochemical properties, where these attributes could be positively or negatively correlated to the moisture content, salt concentration and viscosity of the fermented product.

11.5.4 Value-Added Products from Fermented Marine Animals/Organisms

Research over the decades has always emphasized on the marine animals as reservoirs of resources of human benefit. This not only remains confined to their use as food but also to wide range of value-added products that can be extracted from whole marine animals and their waste. These value-added products could be enzymes, peptides, fats and oils, vitamins, minerals, etc. Table 11.2 provides the names of some fermented products of proven value-added properties. They might also provide additional health benefits due to which they are of increased importance in pharmaceutical, nutraceutical and functional food industries. These value-added products could be derived either from the waste that is generated during the processing of marine-based products or from the fermented products itself.

Fermented of different marine organisms like fish, sharks, shrimps, squids, etc. into products like paste and sauces bring about major changes in the fish proteins by both endogenous enzymes present in the fish or microbial degradation. Enzymes like pepsin, trypsin, carboxypeptidase, amylases and lipase present in the fish starts acting on the flesh as soon as the fish is dead. Also, wide range microorganism present on the flesh or from external solvents like brines of different salt concentration start acting upon the fish proteins into simpler molecules that could then not be spoiled by the degrading microorganisms. It is this process of fermentation that forms key to the preservation of any fish-based product increasing their storage life. During this process, a wide range of compounds are formed that provide the product with specific flavour and aroma. Many of these like alcohols, aldehydes and acids have

Table 11.2 Value-added properties of some marine fermented products

Marine-organisms-based product	Value-added component	Function	References
Anchovy sauce	Angiotensin Converted Enzyme (ACE-I) inhibitor peptides	Tendency to lower blood pressure	Ichimura, Hu, Aita, & Maruyama (2003)
Anchovy sauce	Hydrophobic peptide fraction	Induction of apoptosis in human lymphoma cell line U937	Lee et al. (2003)
Marine blue mussel sauce	Mussel-derived Radical Scavenging Peptides (MRSP)	Antioxidant and radical scavenging properties	Rajapakse et al. (2005)
Fermented Oyster sauce	ACE-Inhibitor peptide	Antihypertensive effect in hypertensive rats	Je, Park, Jung, Park, & Kim (2005)
Shrimp paste (Kapi Ta Dam and Kapi Ta Deang)	Dipeptides (Ser-Val, Ile-Phe; Trp-Pro)	ACE-Inhibitory activity; radical scavenging activity	Kleekayai et al. (2015)
Seacure® (Fermented fish protein concentrate)	Small peptides	Capacity to enhance non-specific host defence mechanism	Duarte, Vinderola, Ritz, Perdigón, & Matar (2006)

already been discussed in the previous sections. Free amino acids, peptides, dipeptides and oligopeptides are formed during the fermentation of fish products that possess antihypertensive, anticoagulant, antioxidant and anticancerous properties.

Researchers have further identified certain other compounds in some of the fish-based products that possess certain properties in additions to their nutritive importance. One such product is a fermented fish-based product commercially known as Seacure® which is a dried fish protein concentrate (Duarte et al., 2006). It is produced by controlled proteolytic fermentation of pacific whiting (*Merluccius productus*) by yeast. This product is found to introduce biological gut repair and integrity in rat model (Fitzgerald et al., 2005). In another study, hydrophobic peptide fractions separated from anchovy fish sauce have been studied for their anticarcinogenic properties. These peptide fractions were studied for their role in induction of apoptosis of cancer cells in human lymphoma cell lines (U937) indicating their cancer chemopreventive effects (Lee, Kim, Lee, Kim, & Lee, 2003). A similar study on marine blue mussel sauce identified peptides with strong scavenging effects on radicals and their antioxidant properties against free radicals (Rajapakse, Mendis, Jung, Je, & Kim, 2005).

Research has also shown that the production of these value-added compounds also vary depending upon the duration of fermentation. For instance, a study on *Kapi*, a traditional fermented Shrimp sauce, found that prolonged fermentation throughout the first 8 months could result in accumulation of short chain peptides and amino acids and Millard reaction products that help in enhancing the antioxidant properties (Faithong & Benjakul, 2014). Another research studies the antioxidant activities of three Thai traditional fermented shrimp products *Kapi*, *Jaloo* and *Koong-Som* (Faithong, Benjakul, Phatcharat, & Binsan, 2010). These works testify the

value-added properties of marine-based products which are mainly due to the fermentation process over period of time that introduces changes in the native proteins, resulting in a wide range of compounds. Since microbial cells tolerant to high salt concentration, also contribute to the fermentation process. The growth of these microorganisms over the period of fermentation releases several important proteins and enzymes. *Jeotgal* is one such fermented fish product in Korea, that serves as the substrate for the production of β-1, 3-1, 4-glucanase, an extracellular protease by *Bacillus spp*. (Kim, Kim, Kim, Choi, & Kong, 2009).

Besides the final fermented products, the waste generated during the processing of marine-based products is another major source of value-added products. Huge tonnes of waste generated from the processing units are already a great cause of concern across all the countries of the world. In order to deal with this problem of waste disposal, several treatment measures are being worked upon. These treatment plants further costs extra amounts to the fishery sector. However, there are enough evidences suggest that this waste is still rich in high value compounds. Shrimp waste comprising of head and shell, for instance is rich in amino acids, peptides, proteins and other nutrients with bioactive properties (Dey & Dora, 2014). Chitin, chitosan and protein hydrolysates have been successfully extracted from shrimp waste (Manni, Ghorbel-Bellaaj, Jellouli, Younes, & Nasri, 2010).

11.6 Marine Plant-Based Fermented Products

Marine agriculture is one of the fast developing sectors of the world food production and international economy with 26.1 million tonnes of aquatic algae produced in 2013 worldwide by 33 countries and territories, and 13.5 million tonnes of aquatic algae is produce yearly in China along. The dominating cultivated marine plants are *Eucheuma* seaweeds (*Kappaphycus alvarezii* and *Eucheuma* sp.) followed by Japanese kelp, or kombu (*Laminaria japonica*), *Gracilaria* sp., Wakame (*Undaria pinnatifida*), *Porphyra* sp. and other seaweeds and microalgae. Filamentous algae (*Spirulina*, *Spirogyra*, *Cladophora* and *Hydrodictyon*), seaweeds (*Ulva lactuca, Gracilaria* sp. *and Porphyra tenera*), floating aquatic macrophytes (*Azolla* sp., *Ipomoea aquatica, Pistia stratiotes, Salvinia* sp. *and Hydrocharis dubia)*, duckweed which is including 37 species belonging to the four genera, water hyacinths (*Eichhornia crassipes*), as well as submerged and emergent aquatic macrophytes are well-known for its exceptional nutritional value and economic importance, and effectively used as natural fertilizers, animal and aqua feeds, source of rare ingredients used in medicine, skincare and cosmetics.

Algae are an excellent source of the natural compounds which have a huge variety of applications in different industries. They are excellent source of proteins and nutrients (Chlorella, Spirulina, Kelp), as well as pigments, lipids (omega-3 (ω3) and omega-6 (ω6)), carbohydrates, macroalgal polysaccharides (agar, alginates and carrageenans) and vital minerals. The algae lipids include acylglycerols, free fatty acids (FFA), phospholipids and glycolipids, and fatty acids biosynthesis is carried

out by biochemical pathways for *acetyl*-CoA production. Microalgae are also an excellent source of high-quality polyunsaturated fatty acids (PUFAs), such as α-Linolenic, docosapentaenoic, docosahexaenoic (DHA) and eicosapentaenoic acids (EPA; ω-3), γ-Linolenic and arachidonic acids (ARA; ω-6). The long chain ω-3 PUFA which are healthy development of the foetal brain (ARA and DHA), they are also effective in reduction cardiac diseases (high blood pressure, stroke and arrhythmia), depression, arthritis, asthma, Crohn's disease, ulcerative colitis, psoriasis, lupus, cystic fibrosis and cancer, especially EPA (Pulz & Gross, 2004).

Algin derived from *L. japonica* is also widely used in manufacturing of plastics, rubber products, pesticides, paints and paper. Some of the red, green and brown seaweeds and some filamentous algae are shown to be an excellent source of vitamins A, B, C, D, calcium, magnesium, potassium iodine, sulphur, selenium and zinc, proteins, algin, agar, chlorophyll, etc., super nutritious food additives for human diet. Many aquatic plants used in production of biofuel, hydrogen, paper (cellulose and hemi-cellulose), building material, animal and aquatic feeds, marine ingredients supply and medical use. One of the most popular marine plant product is seaweed, which is a great source of polyphenols, peptides and polysaccharides (Zhang et al., 2007), extracted from plant using variety of methods. The diagram of the basic products manufactured from the seaweed using different methods (extraction, fermentation and pyrolysis) is shown in Fig. 11.1.

Marine algae by-products obtained using fermentation process using different types of microbes, such as *Bacillus subtilis, Pediococcus acidilacti, P. pentosaceus,* etc. The seaweed fermentation showed an increase in immunoglobulin concentration and stimulation of the immune system in poultry and mammals (Allen & Pond, 2002). Many active compounds derived from seaweed exhibit anticoagulant, anti-inflammatory, antiviral and anticancer properties. Usually, the basic marine plants fermentation process is triggered in distilled water supplemented with yeast extract (0.1 %) and glucose (0.5 %), followed by hydrolysis using cellulose enzyme (4 %), and incubation under static condition. Cosmeceuticals, a novel class of products, which is a combination of cosmetics and pharmaceuticals, include extracts made from algae, seaweeds and sea minerals often possess UV some antioxidant protection properties. A variety of bioactive compounds isolated from marine macroalgae have many great health benefits and demonstrate antioxidant, antibacterial, antiviral, anti-inflammatory, apoptotic and anticoagulant activity, as well as present rich source of water-soluble dietary fibre (50–85 % dry weight). The polysaccharides, such as fucoidan, alginate, laminarin and others derived from marine macroalgae, used as a prebiotic in animal feed and human health products. *Digenea* is an effective vermifuge agent, red algae from family *Dumontiaceae* inhibit the *Herpes simplex virus* and carrageenans have been patented as antiviral agents. *Kelp, Saccharina and Sargassum* polysaccharides could be effective against the breast and other types of cancer, as well as protect from heavy metal toxicity (Andrade et al., 2010). Fucoidans from *Ascophyllum nodosum*, *Saccharina japonica*, *U. pinnatifida, Alaria* sp. and *Fucus evanescens* demonstrate powerful antitumorigenic potential (Vishchuk, Ermakova, & Zvyagintseva, 2013). To obtain the bioactives from the marine plants, researchers used acid– base hydrolysis, hot water or solvent extraction and enzymatic digestion (Ekanayake et al., 2008).

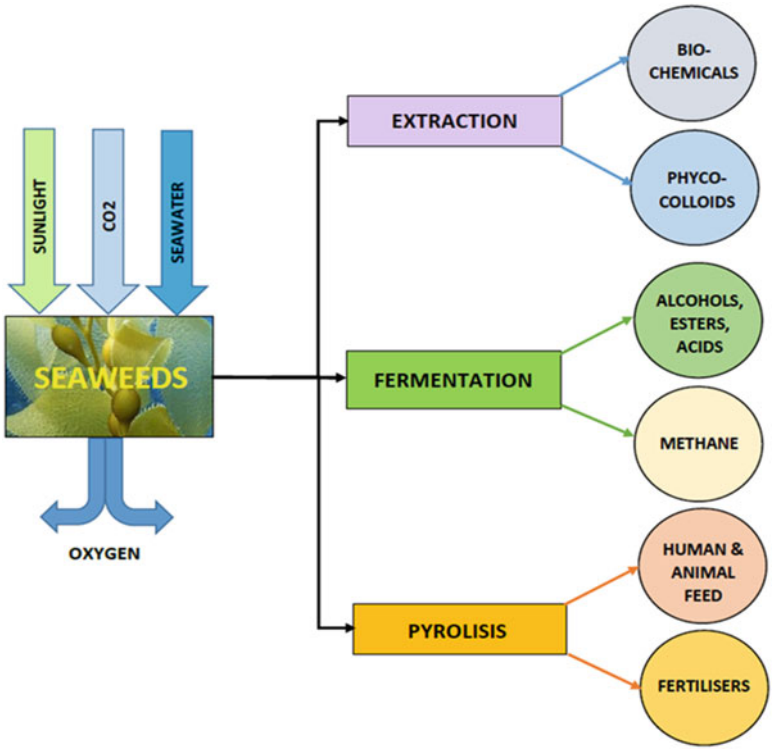

Fig. 11.1 The basic diagram of the types of production from the seaweed plants (Copyright @ by Dr. M. Emerald, 2016)

Pachymeniopsis elliptica, Sargassum horneri and *Ulva pertusa* natural fermentation by adding sugar, water and keeping at room temperature (25 °C) for 3 months has been proposed as a method of active compounds release for potential anticoagulant activity. It has been found that the fermentation process improving the anticoagulant properties of the polysaccharide compound (Ekanayake et al., 2008).

Due to a limited availability of fish oil, and the focus on the use of microalgae as one of the main sources for Omega-3 production, the Omega-3 fatty acid market is expected to grow from current 32B to 36B by the end of 2016. Microalgae are also an abundant source of the omega-3 fatty acids which are crucial for a good health, and a variety of microalgal strains from genera *Phaeodactylum, Nannochloropsis, Thraustochytrium* and *Schizochytrium* has highest content of total lipids, over 50 % of dry weight, both EPA and/or DHA. Omega-3 fatty acids (ω-3) are used in the treatment of rheumatoid, Crohn's disease, ulcerative colitis, neurological conditions, psoriasis, asthma, lupus and cystic fibrosis. The fermentation and conversion of the carbohydrate fraction into glucose prior to lipid extraction caused 15 % increase in additional lipids, as well as improved the solvent extractability of lipids from the algae (Trzcinski, Hernandez, & Webb, 2012) (Fig. 11.2).

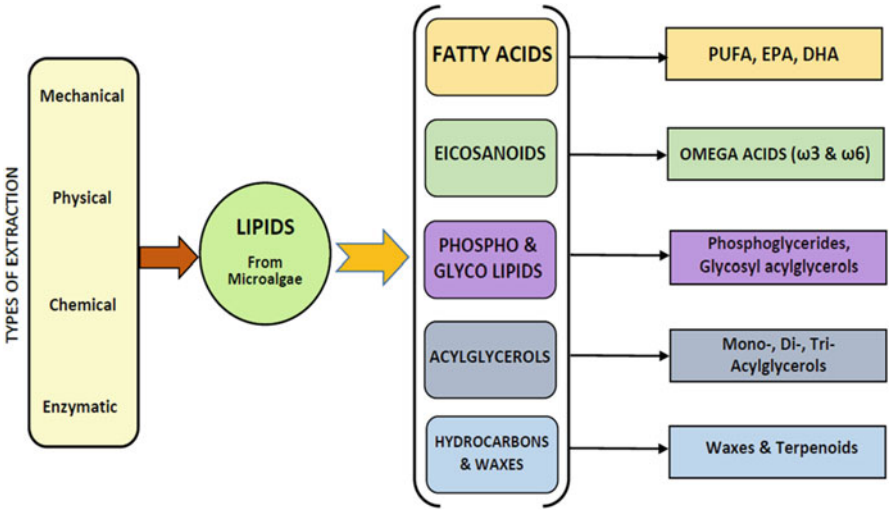

Fig. 11.2 The basic diagram of the types of extraction and final products obtained from the microalgae lipids (Copyright @by Dr. M. Emerald, 2016)

11.6.1 Algal-Based Fermented Food and Value-Added Products

Most fermented foods are made from marine animals like fish and shrimp; however, no food products have yet been developed from marine plant materials like algae. Seaweed *Gracilaria fisheri* has been utilized for the production of a fermented beverage (Prachyakij, Charernjiratrakul, & Kantachote, 2008). Gupta, Abu-Ghannam, and Scannell (2011) used different brown Irish seaweed as sole source of nutrition for LAB and suggested the potential of fermentation of seaweeds with a possibility towards the development of functional food products. The same authors also explored *Saccharina latissima* and *Laminaria digitata* seaweed as a sole source of nutrition for *Lactobacillus Rhamnosus* probiotic bacterium for the development of possible fermented products with health-promoting properties (Gupta, Abu-Ghannam, & Rajauria, 2012). Wijesinghe et al. (2012) fermented the processing by-product of *Ecklonia cava* brown seaweed by the yeast *Candida utilis*. The study demonstrated that fermentation enhanced the polyphenolic content and resultant antioxidant activities, thus suggested fermented *E. cava* processing waste could be a potential alternate for the development of functional food and cosmetic products. Similarly, the red seaweed waste, obtained after extraction of agar-agar, has been fermented with LAB and yeast and the product has been utilized as a fertilizer (Ennouali, Ouhssine, Ouhssine, & Elyachioui, 2006). In another study, Felix and Pradeepa (2012) produced a fermented seaweed-based food for shrimp larvae wherein the *Ulva* sp. of seaweed was fermented with *L. plantarum* (LAB) and food grade *S. cerevisiae* yeast from grape. The substrate was also fortified with potato and soya powder as a sugar and nitrogen substrate, respectively. The findings concluded that the fermented seaweed product was an ideal material for feeding shrimp larvae. The seaweed biomass

is also considered a possible carbon source for lactic acid production which is one of the vital component of food, pharmaceutical, leather and textile sectors (Wee, Kim, & Ryu, 2006). Research showed that sugar from seaweed biomass are more promising or even more beneficial substrate than lignocellulosic biomass for L-lactic acid fermentation and production with different LAB species (Hwang, Lee, Kim, & Lee, 2011). Table 11.3 lists some of the most common algal species and microorganisms used for fermentation for value-added products preparation.

Though, both macroalgae (seaweeds) and microalgae are possible materials for lactic acid fermentation, but their potential have not been explored for any commercial fermented product development. Recent research demonstrated that fermented sauce can be one of the possible products to be developed from *U. pinnatifida* and *Ulva* sp. of seaweed. However, the developed product has limited commercial value because of the shortage of amino acid compounds, which can be explained by the low protein content of seaweed. Additionally, seaweed contains exclusive polysaccharides such as alginate, laminarin, fucoidan and galactan which require novel enzymes to initiate the saccharification process, a very important step of many kinds of fermentations. Therefore, the advancement in enzyme products is essential for saccharification as well as future algal food product development (Uchida, 2011; Uchida & Miyoshi, 2013).

11.6.2 Other Non-food Algal-Based Fermented Products

Apart from food products, algal species have shown a great potential to produce bioenergy (such as biodiesel and bioethanol) through fermentation. The renewable energy production with high energy yields is a vital new direction on the way of prevention and overcome of negative and potentially irreversible effects of fossil fuels and environmental pollution on the global climate with emissions of greenhouse gases. Anaerobic fermentation from various marine macroalgae such as *U. lactuca, P. tenera, U. pinnatifida* and especially *L. japonica* (the great source of carbon) are widely used for production of hydrogen (Mohan, 2010). The standard routes for H_2 production from the algae are based on using fermentation, anaerobic fermentation, enzymatic and microbial electrolysis. The further development of the process including combination of fermentation, microbial electrolysis, bioaugmentation and multiple process integration directed towards improvement of the process efficiency. The most important factors for the water-splitting photosynthesis, photofermentation, dark fermentation and microbial electrolysis directed towards the optimal and effective H_2 production are (a) specific type of algae (e.g. green algae); (b) appropriate substrate and supplementation with nutrients, carbon and nitrates; (c) the optimal concentrations of phosphate; (d) supplementation with suitable metal ions; (e) presence of a specific bacteria for anoxygenic photofermentation (*Allochromatium vinosum, Thiocapsa roseopersicina, Rhodobacter sphaeroides, Chlorobium vibrioforme, Desulfuromonas acetoxidans and Chloroflexus aurantiacus*) (Ghosh, Sobro, & Hallenbeck, 2012) and dark fermentation (*Enterobacter aerogenes, Enterobacter cloacae, Escherichia coli, Citrobacter intermedius,*

Table 11.3 Fermentation of marine plants and microorganisms involved in the process

Raw material	Microorganisms	Value-added component	References
Microalga (*Pavlova lutheri*)	Yeast (*Hansenula polymorpha*)	Peptide for its protective effect against oxidative stress as well as inhibitory effect on melanogenesis	Oh et al. (2015)
Brown seaweed (*Sargassum sp.*)	Marine LAB isolates (*Pediococcus acidilactici, Weissella paramesenteroides Pediococcus pentosaceus*, and *Enterococcus faecium*)	Polyphenol and polysaccharides for antioxidant and anticoagulant activity	Shobharani, Nanishankar, Halami, & Sachindra (2014)
Brown, green and red seaweed (*Undaria pinnatifida, Ulva* sp. and *Porphyra* sp.)	LAB (*Lactobacillus brevis*, *Lactobacillus casei* and *Lactobacillus plantarum*)	Seaweed sauce as a source of protein	Uchida & Miyoshi (2013)
Red and brown seaweed (*Porphyra* sp. and *U. pinnatifida*)	–	Diet for Stockbreeding animals as a source of minerals	Uchida & Miyoshi (2013)
Brown seaweed (*Saccharina latissima* and *Laminaria digitata*)	*Lactobacillus rhamnosus*	Seaweed with higher antioxidant activity for probiotic product development	Gupta et al. (2012)
Brown seaweed (*Ecklonia cava*)	Yeast (*Candida utilis*)	Fermented by-product as a potential antioxidant	Wijesinghe et al. (2012)
Blue-green alga Spirulina (*Arthrospira platensis*)	LAB (*Lactobacillus acidophilus, Bifidobacterium bifidum, L. casei, B. infantis, B. longum, Lactococcus lactis*)	Production of phycocyanin for antioxidant, anti-inflammatory, and UV protective activities	Liu, Hou, Lee, Chuang, & Lin (2011)
Brown seaweed (*Himanthalia elongata, L. digitata* and *Laminaria saccharina*)	*Lactobacillus plantarum*	Fermented seaweed as a source of a range of functional foods	Gupta et al. (2011)
Seaweed sugars	LAB (*L. rhamnosus (KCTC 3237), L. casei, L. brevis, L. diolivorans, L. collinoides, L. salivarius,* and *L. plantarum*)	Biomass feedstock	Hwang et al. (2011)
Green seaweed (*Ulva reticulata*)	*Lactobacillus plantarum* and *Saccharomyces cerevisiae*	Silage preparation for prawn larval development	Felix and Pradeepa (2012)
Brown seaweed (*Sargassum fulvellum*)	–	Anticoagulant activity of sulphated polysaccharide	De Zoysa, Nikapitiya, Jeon, Jee, & Lee (2008)
Brown seaweed (*U. pinnatifida*)	Mixture of LAB (*Lactobacillus brevis*) and yeasts (*Debaryomyces hansenii* and *Candida* sp.)	Marine silage preparation for young pearl oysters	Uchida, Numaguchi, & Murata (2004)

Clostridium beijerinckii, Clostridium paraputrificum, Ruminococcus albus and others). The appropriate substrate and temperature (the maximal yield of H_2 in mixed microbial population is obtained at 35–45 °C) are different and important for different types of bacteria to have a maximum efficiency of the fermentation process. The maximum optimal pH for the efficient H_2 production is maintained at 6.0 (Van Ginkel, Sung, & Lay, 2001). The enzymes (e.g. hydrogenase), co-enzymes and Fe^+ concentrations presented in the substrate are essential for the microbial growth, development and molecular transport, as well as for the effective fermentation process (Lee, Miyahara, & Noike, 2001). The high concentration of Mg^+, Na^+ and Zn^+ were found to be essential to achieve the maximum yields of H_2. The use of the organic substances from the fermentation effluents is a novel proposal. There is also a variety of secondary processes involved, such as methanogenesis, acidogenic fermentation for H_2 and photobiological processes for H_2 production.

The biofuels which can be produced from the algal biomass are biodiesel, bioethanol, biobutanol, biomethane, jet fuel, biohydrogen and thermochemical conversion products such as bio-oil, biocrude and syngas (Chinnasamy, Rao, Bhaskar, Rengasamy, & Singh, 2012). A lot of research is being carried out for developing microalgal biodiesel technology by performing bioprospecting of high-lipid-containing strains as well as by inducing higher lipid production by various physiological and genetic strain improvement methods. Therefore, lipid extraction is an extremely important process for the production of microalgal biodiesel. The cost of microalgae biodiesel is 15.70 US$/L, which is significantly lower than microalgae biodiesel produced from the photobioreactor (73.5 US$/L) (Lam & Lee, 2014). The main adjustments which must be done in order to increase the efficiency, suggesting that an improvement in the fermentation process will reduce the production costs and increase the process efficiency. The main points to pay attention to are the substrate (glucose) feeding and process control in the fermenter must be optimized to reduce the fermentation time frame; the glucose might be changed to a cheaper feedstock, such as cassava, Jerusalem artichoke or waste molasses and high-value by-products or co-products, such as carotene and lutein, might be explored to supplement algal biodiesel production.

Microalgal biodiesel products are usually obtained by bioprospecting of high-lipid-containing strains and inducing higher lipid production by various strains. There are quite a few methods of the lipids and oil extraction from algae, which are established on the present market: Folch, Bligh, Dyer and Matyas methods, superior solvent extraction, lipid hydrolysis, supercritical in situ transesterification, ultrasonic, bead beating, expeller press, solvent extraction, osmotic pressure, isotonic solution, enzymatic and microwave extraction. The solvent extraction, osmotic pressure and isotonic solution are the simplest, economical and sustainable methods with many advantages, such as use of dry and wet algae biomass, free of toxic organic solvents. However, the combinative method (e.g. enzymatic and mechanical, and others) is the most promising and effective lipid extraction which reduces energy consumption and also increases total process efficiency.

There is a huge demand in additional research and development directed towards optimization, better efficiency and process improvement. One of the challenges is high energy consumption in the biofuel production process from the aquatic plants, especially involved into separation of the algal products from the aqueous medium, as well as contribution to greenhouse gas emissions, due to the current energy inputs

required for algal biofuel production. Research dedicated to a low-energy separation processes, use of clear waste water and setting up the nutrients recovery process need to be done in order to increase the effectiveness and to lower the costs of the biofuel production. A major biotechnology advances, such as development of super productive aquatic plants and algae strains and improvement of technical aspects, are needed to achieve sustainable, large-scale algal biofuel production.

11.7 Challenges and Future Trends

Though traditional fermented marine products provide preferred organoleptic quality, but their safety and inadequate shelf-life is still a matter of concern. Since live microorganisms are the integral part of the fermentation, the risk of contamination and consequent toxicity are the major challenges for fermented food products. In addition, excess and regular consumption of fermented food may also expose certain risk and health hazards. According to US federal agency report, Alaskan Eskimos possess high risk of botulism disease because of the consumption of traditional marine-based fermented food. The Eskimos ferment whole fish, fish heads, walrus, seal and whale flippers in airtight plastic container for an prolonged period of time before being consumed. The utilization of airtight plastic containers or wrappers creates anaerobic conditions thus provide favourable environment for *C. botulinum* bacteria to thrive in the microaerophilic conditions which causes botulism (Ganguly, 2012; US Federal Agency Report). Therefore, the fermented food industry is looking for some alternative or synergistic approaches that can enhance the quality and shelf-life of final products without compromising its traditional alikeness. To fulfil the industrial demand, the research has been carried out to explore the application of novel non-thermal approaches like pulse electric field and high pressure processing in last two decades. These emerging techniques offer numerous alternates in developing microbiologically safe with improved shelf-life, healthy and nutritious fermented products with very slight implication on its nutrition and organoleptic characteristics (Ojha et al., 2015; Nordvi et al., 2007).

However, despite the advancement in industrial starter culture and novel process control, the fermented products have not attracted the consumers and none of the production approach has been commercialized yet (Burgess, 2014; Skåra et al., 2015). Although, few producers in Europe is practicing a standardized procedure for fermented fish product preparation but limited scientific knowledge regarding the novel processing techniques is still a biggest challenge for them. Moreover, the complexity of resident microflora and the interaction of different enzymes in gut possess another challenge for its successful implementation (Skåra et al., 2015). Nevertheless, recent advancement in non-destructive spectroscopic analytic tools and improvement in taste and other organoleptic properties could be helpful to expand this knowledge. Furthermore, a deeper knowledge of process monitoring and starter culture will help to control the fermentation and may produce new product types (Skåra et al., 2015; Svensson, Nielsen, & Bro, 2004). Despite the greater advancement in the technology, new industry-based algal fermentation still has a great potential to explore and will remain open for future research.

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Chapter 12
Novel Fermented Grain-Based Products

Mila Emerald, Gaurav Rajauria, and Vikas Kumar

12.1 Introduction

The process of natural fermentation is known earlier than 7000 BC and starts from China (for example, the making of koji, mould -fermented grains and/or soybeans, a source of more than 50 enzymes in has originated in China and was developed in Japan about 1000 years ago) going through different stages of improvement since that time through Georgia, Iran, Babylon, Egypt, Mexico, Sudan and other countries. Grain fermentation is one of the oldest known forms of food preparation, developed thousands of years ago by ancient man with use of one or a few strains of microorganisms to improve digestive and nutritional properties of grains and grains produced beverages (Wood, 1997). However, the deep mechanism of fermentation carried on with microorganisms was not understood and explained properly until about 200 years ago. The first fermented beer is known to be produced since over 7000 years ago in Syria, Mesopotamia and Caucasia and about 5000 years in Babylon, the fermented bread was known from Ancient Egypt and then around 100 BC in Ancient Rome.

M. Emerald (✉)
Phytoceuticals International and Novotek Global Solutions, London, ON, Canada
e-mail: drmilaemerald@gmail.com

G. Rajauria
School of Agriculture and Food Science, University College Dublin, Lyons Research Farm, Celbridge, Co. Kildare, Ireland

V. Kumar
Neuropharmacology Research Laboratory, Department of Pharmaceutics, Indian Institute of Technology, Banaras Hindu University, Varanasi, Uttar Pradesh 221 005, India

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Food grains providing fibres, proteins, vitamins and minerals present over 60 % of the food world production (Charalampopoulos, Wang, Pandiella, & Webb, 2002). The grain components which are hard to digest and such compounds like phytates, tannins and polyphenols (Sindhu & Khetarpaul, 2001) could be broken down by the fermentation process using malt enzymes and microorganisms, especially lactic acid bacteria. This type of fermentation leads to a significant increase in amount of iron, zinc, calcium, vitamin B, essential amino acids, lysine and protein content and improvement of flavour and texture, while decreasing level of complex carbohydrates and mycotoxins (Lee, Lee, Park, Hwang, & Ji, 1999; Nout & Ngoddy, 1997; Yousif & El Tinay, 2000). Sourdough fermentation protects bread from mould and bacterial spoilage, which are especially important in warm natural environment (Corsetti et al., 2000; Rosenquist & Hansen, 1998). Spoilage of sourdough-baked products by mould genera including *Penicillium, Aspergillus, Monilia, Mucor, Endomyces, Cladosporium, Fusarium,* and *Rhizopus* causes a huge economic concern (Legan, 1993). Sourdough fermentation starters produce lactic acid, acetic acid, ethanol, hydrogen peroxide, carbon dioxide and other substances with antifungal properties (De Vuyst & Vandamme, 1994). The fermented grains-based products might have a pleasant appetizing aroma formed by the acetic acid, butyric acid and diacetyl (Blandino, Al-Aseeri, Pandiella, Cantero, & Webb, 2003), and significantly improved digestibility. Grains embryo contain fibre, carbohydrates, proteins, vitamins, sodium, magnesium, calcium, lipids and minerals, and bran layers are rich in proteins, niacin, phytic acid and phosphorus, oligosaccharides and is an excellent source of amino acids for grain fermentations by microorganism. The natural peptides derived by chemical or enzymatic fermentation are reported to possess various beneficial properties including antimicrobial, cholesterol-lowering, antithrombotic, antioxidant properties and cyto- or immunomodulatory effects (Coda, Rizzello, Pinto, & Gobbetti, 2012; Zambrowicz, Timmer, Polanowski, Lubec, & Trziszka, 2013). Fermentation of grains not only improve the yield, but also significantly change the profile of phenolic compounds leading to forming new metabolites through glycosylation, deglycosylation, methylation and glucuronidation, depending on the microbial strains and substrates. These phenolic compounds have antioxidative, anticarcinogenic and anti-inflammatory properties which have potential health benefits for hypertension, obesity, cardiovascular diseases, diabetes and cancer (Celep, Rastmanesh, & Marotta, 2014).

12.2 Grain-Based Fermented Foods and Beverages

There is a wide variety of grain-based fermented products produced by different cultures and in different countries all over the world, using combination of raw or processed grains and other nutritious and tasty ingredients. Among many of them are not only a bread, but also: *koji, tarhana, murri, togwa, ogi, boza, yosa, idli* and *dhokla, sake* and others, which can be classified according to natural substrate used for the fermentation process (Fig. 12.1).

Fig. 12.1 The variety of fermented foods produced internationally using different main substrates: rice, maize, wheat and millet (Copyright @ Dr. M. Emerald, 2016)

Koji (kōji) in Japanese (which is also called 'qu' in Chinese and 'nurukgyun' in Korean), which is first mentioned in 300 BC, is a special traditional national culture made by growing different fungi on cooked grains (Shurtleff & Aoyagi, 2013). The amylases, proteases, lipases and tanninase, bioactive enzymes produced by koji moulds hydrolyzing starches, proteins and fats into glucose, peptides, amino acids and fatty acid. The main fungi used in koji production are *Aspergillus oryzae*, *Aspergillus sojae*, *Aspergillus usami*, *Aspergillus awamori*, *Aspergillus kawachii,*

Rhizopus spp. and *Monascus* spp. Chinese 'qu' fermentations involving different substrates rice, wheat, barley, soybeans and others, as well as fungi genera of *Aspergillus* spp., *Rhizopus* spp., *Monascus* spp., *Mucor* spp. and *Absidia* spp.

Tarhana is Turkish fermented for several (1–7) days at 30 °C with pH 4.3–4.8 mixture which is produced from white wheat flour, yogurt, onions, tomato puree, yeast (baker's yeast), salt, paprika, dill, mint, tarhana otu (*Echinophora sibthorpiana*) and water (Ibanoglu, Ainsworth, Wilson, & Hayes, 1995). The starter culture contains *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Economidou & Steinkraus, 1993). Tarhana is a good source of protein and vitamins and has a sour taste.

Murri is originated in Byzantine, and barley-based represent wrapping of raw barley dough in fig leaves which are left to sit for 40 days with following addition raisins, carob, dill, fennel, nigella, sesame, anise, citron leaf and pine seed.

Togwa produced in Tanzania is fermented beverage prepared from maize, sorghum, millet or cassava. The fermentation could be spontaneous or using *Lactobacillus brevis, Lactobacillus cellobiosus, Lactobacillus fermentum, Lactobacillus plantarum, Pediococcus pentosaceus* and yeasts (*Candida pelliculosa, Candida tropicalis* and *Saccharomyces cerevisiae*) for 9–24 h and pH $\leq$ 3.8 (Mugula, Nnko, Narvhus, & Sørhaug, 2003).

Ogi is naturally fermented product typical for West Africa and could be named such as eko, agidi, kamu, akamu, koko and furah depending on the substrate used. It is produced by lactic acid fermentation of corn, sorghum or millet with occasional addition of soy beans, using *Lactobacillus plantarum*, *Corynebacterium*, *Saccharomyces cerevisiae* and *Candida* spp., by pH 3.6–3.7 (Blandino et al., 2003). Ogi is rich in phosphorous, niacin and riboflavin (Kuboye, 1985).

Boza (which means millet in Farsi language, and known since fourth century BC) is viscous fermented millet, corn, barley, rye, oats or wheat drink. To produce boza, millet is crushed into semolina size pieces and boiled with following addition of water, sugar and starter yeast. After 24 h fermentation at 30 °C, boza is cooled and settled at about 4–5 °C and is ready to be served. This drink contains proteins, carbohydrates, fat and various vitamins; it is high in lactic acid which has stimulating effects on digestion and to the intestinal flora (Arıcı & Turan, 2007).

Yosa is Scandinavian oat bran pudding cooked in water and fermented with lactic acid and Bifidobacteria and flavoured with sucrose, fructose and jam. It is great source of β-glucan, which can reduce the risk of heart disease (Blandino et al., 2003). The starter lactic acid bacteria may belong to the species *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus paraplantarum*, *Lactobacillus fermentum*, *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus salivarius*. Bifidobacteria used are *Bifidobacterium lactis*, *Bifidobacterium longum* and *Bifidobacterium bifidum*.

Idli and Dhokla are a very viscous suspension (batter) made of a blend of rice (*Oryza sativa*) and *Phaseolus mungo*, or other substrate and popular in India and Sri Lanka (Sands & Hankin, 1974). The fermentation is stimulated by *Leuconostoc mesenteroides*, *Streptococcus faecalis*, *Lactobacillus delbrueckii*, *Lactobacillus fermenti*, *Lactobacillus lactis*, and *Pediococcus cerevisiae*, *Lactobacillus mesenteroides*, yeasts *Geotrichum candidum*, *Torulopsis holmii*, *Torulopsis candida* and

Trichosporon pullulans (Chavan & Kadam, 1989). Fermentation increases amino acids and reduces antinutrients (such as phytic acid), enzyme inhibitors and flatus sugars content (Steinkraus, 1983).

12.3 Grain Sourdough and Bread

Sourdough is a mixture of flour and water fermented with lactic acid bacteria and yeasts and first sourdough preparation is known over 5000 years. A huge variety of sourdough products are commercially available (Vogel & Gänzle, 2009). There are many factors which are important for proper sourdough production, such as chemical composition of the raw materials, interactions between the microorganisms, length of fermentation, temperature, dough yield and others. The sourdough fermentation occurs due to its microflora, basically represented by lactic acid bacteria and yeasts (Corsetti & Settanni, 2007). The complex mechanism of sourdough is dependent on the flour used and sourdough microflora. Typical microflora for what bread sourdough is *Lactobacillus*, *Pediococcus, Enterococcus, Lactococcus* and *Leuconostoc*, and *Lactobacillus sanfranciscensis*, *Lactobacillus brevis* and *Lactobacillus plantarum* are the most frequent lactobacilli isolated from sourdoughs (Corsetti, Settanni, Valmorri, Mastrangelo, & Suzzi, 2007; De Vuyst & Vancanneyt, 2007; Hammes & Gänzle, 1998). There are two groups of lactobacillus which are used for food fermentation purposes: homofermentative and heterofermentative. Homofermentative lactobacillus species used in a majority fermented foods for acidification and flavour, which do not produce any carbon dioxide. Heterofermentative lactobacillus species play crucial role in sourdough fermentation, improve taste and flavour of the sourdough breads. Sourdough's with *Saccharomyces cerevisiae* have great stability and resulted in high-profile volatile compounds. There is significant difference between Lactobacillus strains used for production of different sourdough from different regions of the world. Lactic acid bacteria found in Greek traditional wheat sourdoughs produced without yeast, include *Lactobacillus sanfranciscensis*, *Lactobacillus brevis, Lactobacillus paralimentarius* and *Weissella cibaria.* Heterofermentative *Lactobacillus alimentarius* strains are typical for Japanese bread, and *Lactobacillus brevis* and *plantarum* with *Lactobacillus fermentum* found in Russian sourdoughs. Belgian sourdough characterized by *Lactobacillus paralimentarius*, *Lactobacillus sanfranciscensis*, *Lactobacillus plantarum* and/or *Lactobacillus pontis* (Scheirlinck et al., 2007; Murooka & Yamshita, 2008).

According to the production technology and microorganisms, sourdough can be grouped into a three basic types (Fig. 12.2). Type 1, contains pure culture with stable composition, high souring activity and resistant against microbial contamination, which can produce large amounts of lactic acid and acetic acid from maltose (Corsetti, Gobbetti, Rossi, & Damiani, 1998; Damiani et al., 1996). In sourdough, maltose is the most present fermentable carbohydrate, and its catabolism is a key for the proper fermentation process. Sourdough type

Fig. 12.2 Main types of sourdough and its typical microflora (Copyright @ Dr. M. Emerald, 2016)

2 using one-step fermentation which is common now and typical for industrial a large-scale production. This type guarantees more production reliability and flexibility, the microorganisms used have restricted metabolic activity only and are stored fresh up to 1 week (Stolz & Bocker, 1996). Type 3 sourdoughs are initiated by starter cultures available in dry form and mostly contain *Lactobacillus* spp. which are resistant to drying, which is quite simple to use: *Lactobacillus brevis*, *P. pentosaceus* and *Lactobacillus plantarum* strains.

Recently developed commercial dried prepared starter cultures (single- and multiple-strain cultures) consist of *Lactobacillus fructivorans* or *Lactobacillus brevis*, *Lactobacillus pontis*, *Lactobacillus plantarum*, *Lactobacillus delbrueckii* and *Lactobacillus sanfranciscensis* and are quite efficient for the industrial sourdough production (Hamad, Böcker, Vogel, & Hammes, 1992). Powerful antifungal potential of the *Lactobacillus* spp. strains against inhibit *Aspergillus, Fusarium* and *Penicillium*, due to the lactic, acetic and phenyllactic acids, and two cyclic dipeptides (L-Leu–L-Pro and L-Phe–trans-4-OH-L-Pro) have been reported (Gerez, Torino, Rollán, & De Valdez, 2009; Lavermicocca, Valerio, & Visconti, 2003). Combination of *Lactobacillus diolivorans* with *Lactobacillus buchneri* which produced propionate from lactate during sourdough fermentation, as well as addition of powerful antifungal extracts caused significant inhibition of moulds growth for more than 12 days (Coda et al., 2008). However, the application of antifungal *Lactobacillus* spp. cultures in baked products is still limited in spite of their effectiveness.

A variety of yeasts including *Candida milleri*, *Candida holmii*, *Saccharomyces exiguous, Pichia anomala, Hansenula anomala, Saturnispora saitoi, Pichia saitoi, Torulaspora delbrueckii, Debaryomyces hansenii, Pichia membranifaciens* and others, which usually produce metabolites such as alcohols, esters, carbonyl and others, compounds and contribute to leavening, were isolated from sourdough. The type of the yeasts which are active in sourdough formula is dependent on the grain material used, dough hydration, temperature of dough mix, pH, acetic and lactic acid concentration (Gänzle, Häusle, & Hammes, 1997). The bread properties, such as flavour, firmness, crust colour and stickiness, are dependent on the yeast's enzymatic activity caused by proteases, lipases, α-glucosidase, β-fructosidase and other vital enzymes. The bread flavour is composed of volatile and non-volatile compounds, produced both in lactic acid fermentation and in alcoholic fermentation, such as alcohols, ketones, aldehydes, acids, esters, hydrocarbons, ketones, pyrazines, lactones, ethers derivatives and sulphur compounds. Variety of grains and products obtained using the fermentation technologies are powerhouse of phytonutrients and bioactive ingredients which possess significant health benefits. They are rich in vitamins, minerals, dietary fibre, β-glucan, insulin, phytosterols and sphingolipids (Okarter & Liu, 2010) which are crucial for balanced nutrition and human health. Whole-grain phenolic compounds which found in free and soluble forms in corn, wheat, rice, oats, such as phytosterols, ferulic acid, p-coumaric acid, anthocyanidins, quinines, flavonols, chalones, flavones, flavanones, amino phenolic compounds (Adom, Sorrells, & Liu, 2005), usually released via thermal processing and milling provide various health benefits (Chatenoud et al., 1998; Okarter & Liu, 2010). Grains used for bread making also contain lutein, zeaxanthin, β-cryptoxanthin, β-carotene, α-carotene, tocotrienols, tocopherols, oryzanol, unsaturated fatty acids, linoleic acid, oligosaccharides and lignans, which all have important therapeutic properties.

12.4 Grain Brewing

Brewing has been in practice since the beginning of human civilization and nowadays is enjoyed by every ethnic society in the world. Beer is considered the fifth most consumed beverage with an average consumption of 9.6 L/capita (OECD, 2005). The brewing sector holds a strategic economic position which generated a total global revenue of US $318.4 billion in 2014 and is expected to garner US $688.4 billion with 6 % CAGR by 2020 (FAO Source, 2003; Research & Markets Report, 2015). Though the name 'beer' has ambiguity as some people refer it to 'a grain-based fermented beverage', whereas others explain it as a 'hopped drink prepared from liquefied starch after fermentation with specific yeasts' (Meussdoerffer, 2009). The brewing process utilizes barley, wheat or other malted grains along with the adjuncts (unmalted sugar, corn syrups, etc.) and specific strains of *Saccharomyces* yeast to produce beer (Olajire, 2012). The low water activity of stored grains keeps them in metabolically resting state, and therefore, grain constituents are not available for microorganisms, the endogenous enzymes are inactive because of low moisture content and endogenous hydrolytic activities do not take place at this stage. During malting and brewing stage, the water activity of grains increases which continuously change the ecological state of the cereal matrix (Hammes et al., 2005). Though these stages are certainly affected by various characteristic variables which vary from grain to grain and it is necessary to control these basics to get a defined quality product. These variables include the grain type, its nutrients profile and growth factors such as vitamins, minerals and microbial growth inhibiting substance (Hammes & Gänzle, 1998). Among these variables, the type of grain and its carbohydrate contents plays a key role because carbohydrates are a primary substrates and its amount and quality is very important for fermentation (Hammes et al., 2005). After these stages, the substrate becomes available for microorganisms to carry out the fermentation process.

Among all the grains, barley is considered the basic raw material for brewing. It is rich in carbohydrate, protein, dietary fibres, vitamins and minerals. Whole barley grain consists of about 65–68 % starch, 10–17 % protein, 4–9 % β-glucan, 2–3 % free lipids, 1.5–2.5 % minerals, 11–34 % total dietary fibres and from 3 to 20 % soluble dietary fibres of its total mass (Fastnaught, 2001; Gupta, Abu-Ghannam, & Gallaghar, 2010; Quinde, Ullrich, & Baik, 2004). The other non-starch, mixed linkage (1–3 and 1–4) D-glucans and arabinoxylans polysaccharides along with the enzymes help in barley modification for brewing (Gupta et al., 2010). Barley is climatically highly adaptable cereal grain that is produced all over the globe including East and Southeast Asia, the Middle East, North Africa, Northern and Eastern Europe (Newman & Newman, 2006). Figure 12.3 explains the whole brewing process utilizing barley which starts from malting step wherein selected grain goes into an incomplete natural germination process which involves a series of enzymatic degradation and releases the starch from endosperm matrix. This series of structural and biochemical degradation referred to as endosperm modification (Gunkel, Voetz, & Rath, 2002). The malting, comprises steeping, ger-

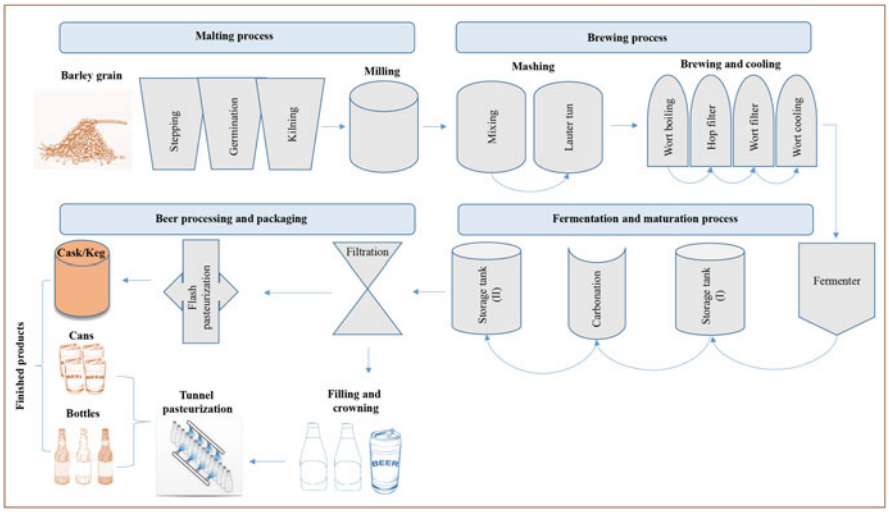

Fig. 12.3 The process of beer production from barley grain

mination and kilning, is a controlled process which ensures the final brewed product quality and stability. Endosperm structure, starch content, starch granule size and distribution, protein content, cell wall properties and endogenous enzymes are few factors that affect the whole malting process (Ogushi et al., 2002). After this step, the enzymatic activity and soluble protein content is increased and more simple sugars are freely available which change the typical colour and flavour of the product (Hoseney, 1994). After completion of milling, the malted grain is transferred to the brew house for mashing which is first but a key step of brewing process. During mashing, the enzymatic degradation of malt takes place and a mixture of milled malt, gelatinized adjunct and water called 'mash' is produced. Furthermore, the starchy content of the mash is hydrolyzed through a series of mixing, heating and cooling process, and a liquid called sweet wort is produced. This step ensures to obtain high yield of sweet wort and also safeguards the end products uniformity (Olajire, 2012). Once the wort is cooled, it is inoculated and mixed with yeast under the influence of oxygen and transferred into the fermenter vessel. The fermenter vessels come in all sizes, big and small ranging from 20 barrel (24 hectolitres) to as large as 6000 barrel (7200 hectolitres) tank depending upon the size of breweries (Gribbins, 2013). During fermentation process, the yeast metabolized the fermentable sugars in the sweet wort and converted it into alcohol (Gupta et al., 2010; Olajire, 2012). In this step, a series of chemical and enzymatic reaction takes place inside the brewing yeast cell which utilizes different sugars (glucose, fructose, sucrose, maltose and maltotriose) depending upon the type of malted grains. The carbon dioxide (CO_2) is also produced during the fermentation step which can be recovered and reused later. Fermentation process time varies from a few days to closer to 10 days and the rate of fermentation depends upon type of the yeast strain, taste profile and other fermentation param-

eters. After the completion of fermentation step, yeast is removed from the vessel and the product is stored in an aging tank. The carbon dioxide produced at this stage is dissolved in the beer and carryout the carbonation process. Beer aging or maturation is the last step wherein the beer is cooled and stored. The settled yeast and other precipitants is removed and the beer is held at conditioning temperature (−1 to 10 °C) for several days to over a month for maturation and stabilization. Finally, the beer is dosed with hop extracts and flavour additives, pasteurized to remove harmful microorganisms and filtered with various types of filters (Olajire, 2012). The filtered beer is then packed into the bottles, can or keg and transported to the pubs/super markets.

The brewing is an energy intensive process and uses large volumes of water, especially in the brew house, where mashing and wort boiling take place (Olajire, 2012). Antioxidant compounds from malted grain or incorporated hop extracts improve the beer flavour stability. The availability of high level of phenolic compounds in malted grains seems very important to produce nutritionally healthier beer with high levels of antioxidant activity (Maillard, Soum, Boivin, & Berset, 1996). Despite the types of grain, the taste of the beer depends upon the whole brewing process including biochemical and microbiological changes that occur during malting, fermentation and subsequent processing and storage throughout the production. For instance, compared to the Western-type beer, traditional African beer tastes more saury, less carbonated and contains no hops. They are usually consumed unrefined which includes unfermented substrates and microorganisms (Blandino et al., 2003; Haggblade & Holzapfel, 1989, 1993).

Though beer is one of the most commonly consumed beverages but, the consumption of conventional grain (barley, wheat and rye)-based beer is not safe for coeliac or gluten-intolerant people (Hager, Taylor, Waters, & Arendt, 2014). In the modern brewing process, the gluten protein and coeliac toxic polypeptides are removed via polyvinylpolypyrrolidone (PVPP) and silica gel filtration process and a very small percentage of total protein/peptides (0.2–0.6 %) remains in beer (Dostalek, Hochel, Mendez, Hernando, & Gabrovska, 2006; Picariello et al., 2011). Nevertheless, very small amount of gluten or coeliac toxic peptide in beer is enough for the immunotoxicity. Therefore, rice, corn, sorghum, millet and pseudocereals (quinoa, buckwheat and amaranth) are considered safe for gluten free beer production (Hager et al., 2014). Apart from health issues, people in Africa and Asia utilize other grains for brewing to get the different taste and flavour. In Africa, malted or germinated single cereal grains or a mixture of them is typically used in *Pito* and *Burukutu* brewing, whereas *Ajon*, *Omuramba* and *Kweete* brewery products are prepared from finger millet, sorghum and a mixture of maize and millet, respectively (Iwuoha & Eke, 1996; Mwesigye & Okurut, 1995). Furthermore, rice-based fermented brewery products such as *Makgeolli*, *Mijiu*, *Pangasi*, *Raksi*, *Rượu cần*, *Sake*, *Sato*, *Shaoxing*, *Sombai*, *Sonti*, *Tapai*, *Takju*, *Tapuy*, *Brem*, *Tuak*, *Ara*, *Cheongju*, *Cholai*, *Choujiu*, *Huangjiu*, *Hariya* and *Lao-Lao* are very popular in East and Southeast Asian countries (Banigo & Muller, 1972; Sankaran, 1998; Steinkraus, 1998; Svanberg & Sandberg, 1988).

12.5 Grain-Based Fermentation: Global Trends and Frontiers

According to the UN Food and Agriculture Organization, over 795 million people (majority is from the developing countries) are suffering from malnourishment and hunger in 2014–2016. In 2000, world leaders set out the Millennium Development Goals (MDGs) reflecting the world's commitment to improve the lives of people and beat the hunger. The term undernourishment defines insufficient food intake as the consumption of less than 1600–2000 calories per day. Sub-Saharan Africa represents about 58 % of the total food-insecure population (Rosen, Meade, Fuglie, & Rada, 2014). Taking into consideration that grains cultivation and harvesting are the main focus in many regions of the world, there is a strong trend on towards development of new nutritious and effective human and animal feeding products, especially fermented foods. Fermentation of grains transforms the flavour of food, its digestibility, shelf-life, texture and taste, adding beneficial bacteria and enhanced micronutrients which are crucial for human and animal health and proper nutrition. Fermented food in general, and fermented grains-based products have earned a great international reputation for the beneficial effects on immunity, which is especially important not only for the developing countries, but also for the intestinal health and general well-being. Lactic acid fermentation increases production of amino acids, especially lysine, which has powerful antiviral properties. It is especially important for the regions of the world where medical help is rarely available and where population is suffering from many rare and contagious diseases. Grains fermentation is crucial for reduction of phytic acid, non-nutrient which is typical for the unprocessed grains, and which binds up minerals and preventing their full absorption. The lactic acid fermentation enables better mineral absorption from the grains and results in better quality of food. A variety of bread produced from sourdough using different microorganisms and yeasts is an important solution which can help eliminate the hunger and nutritional deficiencies. Making fermented porridges from the grains is very popular in Africa and is the best available food for babies. Malted grains are rich in enzymes (amylase) that digests the starch into sugars which dissolve in the water. Fermentation makes the food more nutritious, and there are quite few new substrates which can be discovered and used, such as quinoa, buckwheat, chia and others. The traditional grains fermentation can reduce mycotoxin transfer from grains to fermented foods and increases food nutritional profile via synthesis of protein, vitamins and amino acids. There is much more research need to be done to understand further and discover the result of the bacterial diversity during grains fermentation, and to determine the precise mechanism of the mycotoxin reduction due to the fermentation process.

Countries all over the globe support production and use of biofuels produced via complex fermentation process from the grain products and animal feeds by-products, which will be leading to the domestic energy source, economic development and reduction of the emissions of greenhouse gases. The two primary biofuels produced globally today (ethanol and biodiesel) are derived from grain, sugar and

oilseeds, and use of certain feedstocks for biofuels production also results in the co-production of animal feed. Biofuel production can be increased via increasing crop yields, and availability of the grain substrate for the ongoing manufacturing. One of the problems is the lack of quality measurements for the nutrient characteristics in distiller's grains. Distillers grains produced at different plants vary in texture, colour and nutritional profile, and quality. The attractiveness of the grains fermentation-based industry depends on the price levels of the functional molecules and the yield of fermentation processes. Development of crops for biomaterials, biochemicals and nutraceuticals is very important for economical growth and our society, and there is a lot of research and further development needs to be done in order to produce spoilage-resistant bread, better nutritional quality food and beverages, as well as clean ethanol and more affordable and efficient biofuel.

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Chapter 13
Novel Fermented Fruit and Vegetable-Based Products

Raffaella Di Cagno, Pasquale Filannino, and Marco Gobbetti

13.1 Plant-Based Functional Products

Functional foods development represents one of the most innovative trends in food industry. Between 2005 and 2009, the global launches of functional products have been reported more than doubled, from 904 to 1859 (Valls et al., 2013). Fermented dairy products are the most common vehicles for functional components, though the research is driven toward plant-based functional foods, as the consequence of the ongoing trend of vegetarianism, the level of cholesterol of dairy products, and the increasing prevalence of the lactose intolerance. Fermentation represents a valuable biotechnology to improve nutritional and functional features of plant material. In this overview, several strategies have been implemented in order to develop novel fermented plant-based functional foods. Microorganism functionality may be exploited to increase bioactive compounds during fermentation. Innovation also involves the testing of novel formulation with natural ingredients or by-products food industries as functional ingredients. Moreover, foods may be fortified with probiotics and prebiotics.

13.1.1 Microorganism as Cell Factories for Functional Food Development

Bioprocessing of plant materials using bacteria (e.g., lactic acid bacteria, bifidobacteria, *Bacillus* spp., and *Gluconobacter* spp.), yeasts (e.g., *Saccharomyces cerevisiae*), and fungi (e.g., *Aspergillus* spp., *Rhizopus* spp., and *Monascus* spp.) provides

R. Di Cagno (✉) • P. Filannino • M. Gobbetti
Department of Soil Plant and Food Sciences, University of Bari Aldo Moro, Bari 70126, Italy
e-mail: raffaella.dicagno@uniba.it; pasquale.filannino1@uniba.it; marco.gobbetti@uniba.it

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strategies to produce bioactive compounds and enhance food nutraceutical properties. Microorganisms may improve bioavailability of vitamins, minerals, amino acids (e.g., γ-aminobutyric acid), and phytochemicals (e.g., phenolics and sterols) as well as lead to a marked increase of microbial metabolites (e.g., organic acids, exopolysaccharides, and conjugated linoleic acids) (Frédéric & De Vuyst, 2014).

A common application of fermentation is the manufacture of foods enriched with antioxidant compounds. Several phenolic derivatives showed higher antioxidant property than their precursors after microbial bioconversion. Besides, bioconversion of phenolic compounds, fermentation may also promote other alterations in the food properties, with effect on human health. Fermented cherry and pomegranate juices, broccoli puree, cowpeas, and onions have been enriched in phenolic derivatives with high bioavailability, due to bioconversion using selected *Lactobacillus* spp. (Di Cagno, Surico, et al., 2011; Filannino et al., 2013; Filannino, Bai, Di Cagno, Gobbetti, & Gänzle, 2015). In fact, it was found that phenolic aglycones have higher antioxidant activity and human bioavailability than their glycosides. Nevertheless, human tissues and biological fluids do not possess esterases, capable of hydrolyzing phenolic glycosides, and only the colonic microbiota would be capable of carrying out this hydrolysis (Manach, Scalbert, Morand, Rémés, & Jiménez, 2004). Phenolic esterases are widespread in *Lactobacillus* spp., and allow to metabolize phenolic glycosides that are abundant in plant matrices, improving the inherent functional value of fermented fruits and vegetables. Also the fermentation of mung beans by *Rhizopus oligosporus* was shown as being able to mobilize the conjugate forms of phenolics and improves their health-linked functionality (Randhir & Shetty, 2007).

A further approach includes the release of bioactive peptides exploiting the proteolytic system of microorganisms. Plant proteins have been less studied than animal proteins, though they may represent an excellent source of bioactive peptides with antihypertensive, antioxidative, anti-obesity, immunomodulatory, antidiabetic, hypocholesterolemic, or anticancer properties. Plant peptides include hypogin (peanut), angularin (adzuki bean), lunasin (soybean and barley), Bowman–Birk inhibitors (soybean and lentil), and trypsin inhibitors (mustard) (Kadam, Tiwari, Álvarez, & O'Donnell, 2015).

Fermentation also represents an effective biotechnological approach for production/extraction of microbial metabolites useful for pharmaceutical purpose or as food additives. A promising strategy is the use of by-products as a source of active ingredients to produce functional foods. By-products generated during agricultural production or industrial food processing (e.g., pomace, seeds, peels, straw, sugarcane bagasse, corn stover, cobs, and husks) contain soluble sugars, fiber, proteins, and polyphenols that may be metabolized by a range of microorganisms into valuable bioactive compounds. Protein-rich by-products may represent a valuable substrate for peptide-based functional food development. The bioconversion of lignocellulosic materials for the production and extraction of bioactive phenolic compounds can also be considered. Recently, pomegranate husks, green coconut husk, and cranberry pomace were successfully used as fruitful sources for ellagic and ferulic acids and other phenolic compounds through bioprocessing with *Aspergillus niger*, *Lentinus edodes*, and *Phanerochaete chrysosporium*. Enzymes such as α-amylase, laccase,

β-glucosidase, tannin acyl hydrolase, and ellagitannin acyl hydrolase, among others, play a key role in bioprocessing of lignocellulosic substrates. Though the enzymes involved are mainly released by fungi, the enzymology of bacterial lignin breakdown is currently underway. *Bacillus* sp. strains were positively evaluated for their alkali lignin-degrading ability. *Lactobacillus pentosus*, *Pediococcus pentosaceus,* and *Pediococcus acidilactici* strains were also proposed for efficient bioconversions of lignocellulosic feedstock. The advantage of harnessing the biosynthetic ability of bacteria rather than fungi is that bacteria are better suited to implementation of genetic engineering strategies (Boguta, Bringel, Martinussen, & Jensen, 2014; Chang, Choi, Takamizawa, & Kikuchi, 2014; Martins et al., 2011).

Recently, wastes and by-products occurring in the food supply chain have received attention to produce single-cell protein (SCP) as protein supplement for both human food and animal feeds. New sources of protein can alleviate the world's protein deficit occurring from conventional protein sources. Lignocellulosic biomass is rich in fiber and fermentable sugars, but low in protein content. Microorganisms, such as filamentous fungi, yeast, and bacteria, may be involved in bioconversion of by-products (e.g., pomace, peels, straw, corn stover, cobs, and husks) to produce biomass rich in proteins and amino acids. As plant proteins are generally low in lysine, a major advantage of SCP is its high lysine content. Microorganisms selected for this purpose have to show a high specific growth rate and biomass yield, and a high affinity for the substrate with a low requirement for growth factor supplementation. Yeast such as *Kluyveromyces* spp., *Candida* spp., and *S. cerevisiae* were successfully used in SCP production. Filamentous fungi have received great attention due to their ability to use a large number of complex growth substances such as cellulose and lignin. Cellulose is the most abundant organic compound on earth. Bacteria and fungi may synthesize cellulases under aerobic or anaerobic conditions.

Compared to monocultures, mixed cultures may lead to better substrate utilization and increased productivity due to the synergistic interactions between compatible microorganisms (Gutierrez-Correa, Portal, Moreno, & Tengerdy, 1999). For instance, coculture of cellulolytic moulds (e.g., *Aspergillus niger*) and yeasts (e.g., *Candida tropicalis*) has shown an increase in protein content of apple pomace probably because fermentable sugars obtained from cellulolytic mould and the yeast would have used sugars released (Bhalla & Joshi, 1994).

Fermentation may improve the nutritional properties of foods by removing antinutrients (e.g., oxalate, protease and α-amylase inhibitors, lectins, condensed tannins, and phytic acid) and harmful components (e.g., mycotoxins, biogenic amines, and cyanogenic glycosides). In order to reduce mycotoxins levels in foods and feeds, degradation by microorganisms (e.g., *Saccharomyces* spp., *Acinetobacter calcoaceticus*, lactic acid bacteria, bifidobacteria, or *Aspergillus* spp.) was reported in literature as an effective strategy (Bejaoui, Mathieu, Taillandier, & Lebrihi, 2004). Further examples include the removal of raffinose, stachyose, and verbascose in soy, to prevent flatulence and intestinal cramps, and the decrease in levels of proteinase inhibitors in legumes, to improve the protein digestibility.

13.1.2 Novel Formulations with Natural Ingredients

New product development is a constant challenge for functional foods sector. Beneficial synergies among food ingredients, food formulation, and processing methods can be an effective strategy for food innovation. Recently, a novel protocol for pilot scale production of fermented cherry puree was proposed by addition of stem infusion (10 %, v/v) to improve the phenolic profile (Di Cagno, Surico, et al., 2011). The selection of the best formulation is a crucial step for the design and development of novel foods in order to obtain suitable physicochemical, functional, and sensorial attributes with extended shelf-life, chemical stability, and reasonable price. A critical factor to be considered is the knowledge of the interactions that might occur when several ingredients are mixed together. These ingredients may undergo to various chemical and/or physical changes (e.g., precipitation, oxidation, insolubility, and degradation) and may affect the survival of microorganisms. The selection of the growth substrate should be based on the nutritional requirements of microorganisms, and in some cases supplementation of substrate may be required. Fermentation of mixed substrates may have advantages such as the possibility to develop a complete fermentation process without need for extra nutrients. A recent study aimed at investigating the capacity of selected lactic acid bacteria to enhance functional features of *Echinacea purpurea* with the prospect of its application as functional food (Rizzello et al., 2013). Since *Echinacea* powder suspension in distilled water allowed a very poor growth of *L. plantarum* starters. Yeast extract or grape must were added to optimize bacterial growth conditions. Grape must was also used as a substrate to synthesize γ-aminobutyric acid (GABA) by *L. plantarum* for the manufacture of a functional beverage (Di Cagno, Mazzacane, et al., 2010).

13.1.3 Fortification of Fermented Fruits and Vegetables with Probiotics

There is a strong interest toward the development of fermented plant-based functional products fortified with probiotics. Most probiotics conveyed through fermented foods and food complements belong to *Bifidobacterium* and low GC (guanine and cytosine) percentage lactic acid bacteria. The use of yeast as a probiotic food supplement is still restricted and is not completely elucidated, although they represent a significant part of the characteristic microbiota of several traditional fermented products associated to health benefits. To date, *Saccharomyces boulardii* is the main recognized probiotic yeast. More than other food matrices, raw fruits and vegetables may represent the ideal vehicle for functional health ingredients, since they are inherently rich in beneficial nutrients and have a microstructure comprised of sites (e.g., intercellular spaces, stomas, lenticels, capillaries, irregularities naturally occurring, and tissue lesions), which favors the microbial internalization and protection. Moreover, indigestible molecules (e.g., fiber, inulin, and fructo-oligosaccharides)

protect probiotic microorganisms from the acidic environment of the stomach and are a source of nutrients, which positively influences bacterial survival. However, the lack of certain vitamins and essential amino acids in some plant species may limit the growth of probiotics. Microbial viability and functionality is strongly dependent on the strain and on the nature of plant substrate. Sheehan, Ross, and Fitzgerald (2007) reported significant differences with respect to the acid resistance property of *Lactobacillus* spp. and *Bifidobacterium* spp. in orange, pineapple, and cranberry juices. All of the strains screened survived for longer in orange and pineapple juice than in cranberry juice, and few *Lactobacillus casei*, *Lactobacillus rhamnosus*, and *Lactobacillus paracasei* strains showed the greatest robustness. Although probiotic bacterial strains are currently isolated and characterized mainly from the human gastrointestinal tract, raw fruits and vegetables may represent an alternative source of novel probiotic candidates able to survive to gastric and intestinal fluids, capable of adhering to the gut epithelium and exerting a beneficial effect on the health of the host (Vitali et al., 2012). Inherent chemical and physical features of raw fruits and vegetables mimic those of the human gastrointestinal tract (e.g., extremely acid environment, high osmotic pressure, poor nutrient profile, and presence of indigestible nutrients and antibacterial compounds). Thus, adaptation to the harsh environmental conditions of plant matrices makes plant-derived bacteria capable of reaching the intestine in the living state. Several works showed that the resistance ratio of plant-derived lactic acid bacteria to gastric juices and bile may be comparable or even higher than animal-derived lactic acid bacteria.

13.2 Microbial Starter Selection for Novel Fermented Plant-Based Foods

Today, lactic acid bacteria play a prominent role in the world food supply, performing the main bioconversions in fermented products. For the manufacturing of commercial novel fermented plant-based foods, spontaneous fermentation with unsterilized raw vegetables and fruits leads to the growth of various lactic acid bacteria, which makes it difficult to control the fermentation process. In some cases, the alcoholic fermentation takes place concomitantly. Overall, the spontaneous fermentation of vegetables and fruits includes the succession of hetero- and homofermentative lactic acid bacteria, together with or without yeasts (Plengvidhya, Breidt, & Fleming, 2004). Notwithstanding the reliable value of the spontaneous fermentation to stabilize and preserve raw vegetables and fruits (e.g., cucumbers, onions, eggplants, red-beets, capers, lychee, cocoa beans, and persimmon), a number of factors are in favor of the use of selected starters. Some of these factors include the risk of fermentation failure, the inadequate inhibition of spoilage and pathogen microorganisms, and the undesirable and unpredictable variations in the sensory, nutritional, and rheology properties (Di Cagno, Coda, De Angelis, & Gobbetti, 2013). The use of starter cultures was considered as an alternative to address these

outstanding drawbacks. Recent trends suggest that the demand for starter cultures is on the rise. Overall, two main options may be pursued for the controlled lactic acid fermentation of fruits and vegetables include the use of autochthonous or allochthonous starters (Di Cagno et al., 2013). Autochthonous starter means isolated from and reused on the same raw matrix, apart from the geographical origin. Allochthonous starter means isolated from certain raw matrices but used to ferment various products. Obviously, commercial starters, which are used to ferment a variety of vegetables and fruits, mostly coincide with the above definition of allochthonous strains. Authorized lists of microorganisms with certified use in food fermentations, which cover a wide range of food matrices, including vegetables and fruits, were recently published (Bourdichon et al., 2012). These lists may represent a de facto reference of food cultures, which should be consulted to select starter for fermentation of raw vegetables and fruits. Usually, commercial starters are not previously selected to ferment a specific vegetable or fruit matrix and in some cases, may present several limitations (1) the selection did not consider other features than rapid acidification; (2) the adaptation to the main sensory and functional properties of the matrix is poor; (3) the metabolic flexibility is low; and (4) the diversity did not reflect the ecosystem where they have to be used. Consequently, highly performing commercial/allochthonous starter cultures are very rare. Selection of starter cultures within the autochthonous microbiota of fruits and vegetables should be recommended since autochthonous cultures may ensure prolonged shelf-life and targeted nutritional, rheology, and sensory properties. Indeed, autochthonous strains always had better performances than commercial/allochthonous strains. Not all the strains that compose the lactic acid bacteria microbiota of vegetables and fruits may guarantee the same performance during processing. Therefore, their selection is indispensable. The main criteria for selecting starters to be used for vegetable fermentation are the (1) rate of growth; (2) rate and total production of acids which, in turn, affects the changes of pH; and (3) environmental adaptation/tolerance. Studies on starters to be used for plant-based foods have mainly focused on the production of products with extension of the optimal ripening period, improved sensory features, functionality, and enhanced safety by using predominant lactic acid bacteria, acid-resistant strains inhibiting overacidifying microorganisms, and bacteriocin-producing strains. Three main criteria for selecting lactic acid bacteria as starters for novel fermented plant-based foods are shown in Fig. 13.1. Predominance of growth by a species of lactic acid bacteria is influenced by the chemical and physical environment in which it has to compete. *L. plantarum*, which predominates the later stage of vegetable fermentation due to its high acid tolerance and metabolic versatility, seems a likely choice when homolactic fermentation is desired. Moreover, robustness of autochthonous starters throughout fermentation and storage processes are able to achieve high cell numbers (ca. 8.0–9.0 log cfu g^{-1}) is an indispensable prerequisite to ensure both safety and potential probiotic properties of the product. The synthesis of exopolysaccharides is another metabolic trait to be considered for the selection, especially for lacto-juices and -puree. In addition, the capacity of lactic acid bacteria to synthesize protopectinases, which may enhance the viscosity of fruit matrices, may be an important characteristic for starters. Growth and viability

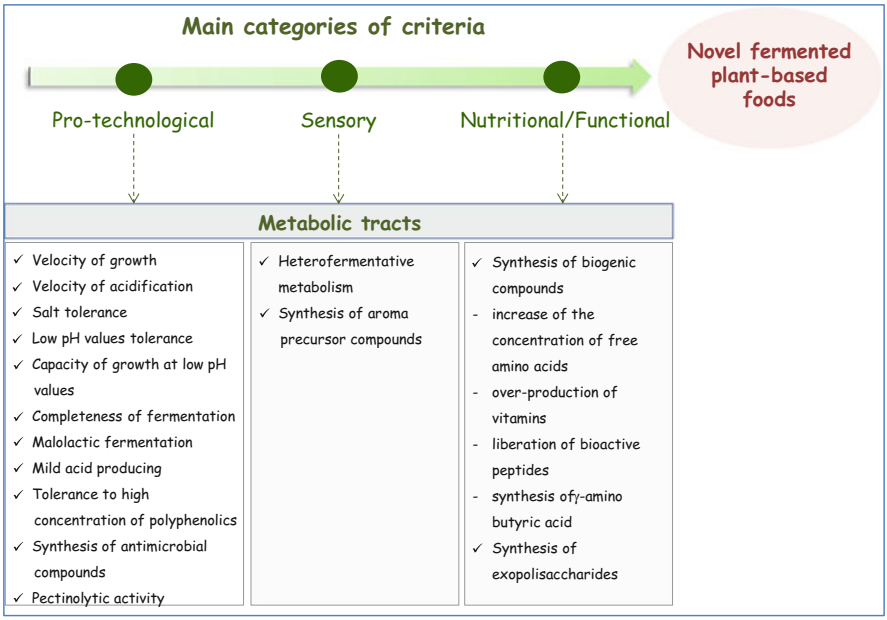

Fig. 13.1 Main criteria for selecting lactic acid bacteria as starters for novel fermented plant-based foods

of lactic acid bacteria, in particular *L. plantarum* and pediococci, are frequently shown on plant materials where polyphenolic compounds are abundant. *L. plantarum* has shown the metabolic capacity to degrade some phenolic compounds and/or other related chemical compounds. Exploitation of bacteriocinogenic lactic acid bacteria on common spoilage and pathogenic microorganisms of raw vegetables and fruits have been extensively investigated. Although nisin is the only purified bacteriocin used thus far in industrially processed foods, many bacteriocins produced by various lactic acid bacteria may have potential applications in biopreservation from common spoilage (yeasts and molds) and pathogenic (e.g., *Listeria innocua*, *Listeria monocytogenes,* and *Escherichia coli*) microorganisms.

13.3 Novel Plant-Based Fermented Products

Rapidly evolving technological capabilities has led to several innovations in plant-based products. These consumer-oriented innovations are mainly related to the ingredient exploration and development. The focus of food product development process is to improve convenience, nutritional, functional, and hedonistic features of fruits and vegetables. Particular attention is addressed toward plant-based functional beverages and fresh-cut minimally processed products.

13.3.1 Plant-Based Beverages

Nowadays, there is a strong tendency toward consumption of fresh-like, highly nutritional value, health-promoting, and rich flavor beverages, for example, fermented juices, smoothies, and yogurt-like product. Nondairy beverages market is supposed to have an annual growth rate of 15 % during the next few years (Marsh, Hill, Ross, & Cotter, 2014). One of the most attractive opportunities is the development of lactic acid-fermented fruit and vegetable juices, also named "lactojuices," which have been shown to have considerable market value and consumer acceptance, as they are perceived as healthy and refreshing beverage. The technological options for the manufacture of fermented vegetable juices mainly include the following three (1) spontaneous fermentation by autochthonous microbiota, (2) fermentation by selected starter cultures added into raw vegetables, and (3) fermentation of mild heat-treated vegetables by starter cultures. Plethora of literature regarding fermented lactojuices is available. Various fruits and vegetables including watermelon, sapodilla, carrot, potato, beetroot, pepper, parsley, lettuce, lemon, cabbage, spinach, tomato, pomegranate, blackcurrant, orange, grapes, sweet potatoes, apple, pear, and cashew apple have been employed for the production of lactojuices. Pomegranate has gained great popularity during the last decade due to the growing scientific evidence for its high nutritional and functional value, thus different fermentation technologies were recently applied to develop novel functional juices (Filannino et al., 2013; Mousavi, Mousavi, Razavi, Emam-Djomeh, & Kiani, 2011). Despite health benefits has been poorly demonstrated with clinical trials or even animal models, in most of cases in vitro studies stated the positive effects of fermentation on the health-promoting properties of juices (e.g., antioxidant, immunomodulation, antihyperglycemia, antihypertensive, ACE inhibitor, antitumor, bile acid-binding, and hemagglutinating activities). Lactic acid fermentation has been successfully used to preserve or improve sensory properties (texture, flavor, and color) of fruit and vegetable juices (Di Cagno et al., 2013). Fermentation by *L. fermentum* has been successfully applied in deacidification of *Prunus mume* fruits, since the fresh fruits are unsuitable for direct consumption due to the high inherent acidity and amygdalin (bitter taste), with a good prospect in the development of probiotic beverage (Yu et al., 2015).

The use of selected starter cultures may also improve juice yield thank to the activity of pectinolytic enzymes. The fermentation by selected lactic acid bacteria has been largely used to enhance the antimicrobial, antioxidant, and immunomodulatory features of several medicinal plants. For instance, an innovative functional and probiotic fermented beverage has been developed using herbal mate fermented by . A major advantage of this product is the compliance to organic claims, while providing caffeine and other phytostimulants without the addition of synthetic components in the formulation (Lima, De Dea Lindner, Soccol, Parada, & Soccol, 2012).

The probiotication of several fruit and vegetable juices with lactic acid bacteria and bifidobacteria was also successfully reported. Several options are currently pursued for improving bacterial viability in plant juices. The addition of yeast autolysate (e.g., spent brewer's yeast) into the juices increases the number of lactic acid

bacteria during the fermentation and reduces the time of fermentation. The prebiotic effect of the cashew apple juice fermented by selected *Leuconostoc mesenteroides* was increased due to the addition of prebiotic oligosaccharides (Vergara, Honorato, Maia, & Rodrigues, 2010). The exposure of bacteria to a sublethal stress might induce a kind of resistance and an adaptive stress response. Cultivation in an acidified laboratory medium (acid stress) or containing vanillic acid (phenol stress) or in a substrate added with variable amounts of fruit juice was proposed as a successful protocol to increase *Lactobacillus reuteri* survival in fruit juices (Perricone, Bevilacqua, Altieri, Sinigaglia, & Corbo, 2015). Furthermore, *L. casei* strains gained higher resistance to simulated gastric digestion after the exposure to the acidic conditions of fruit juices during refrigeration (Céspedes, Cárdenas, Staffolani, Ciappini, & Vinderola, 2013). Stability and sensory acceptance must be considered during the development of probiotic fermented juices (Granato, Branco, Nazzaro, Cruz, & Faria, 2010). Though several novel probiotic juices were found to have good aroma and flavor compared to nonprobiotic juices, however, in some cases unsuitable aromas and flavors may occur. A proper selection of the fruit matrices, probiotic strains, and eventually the addition of other ingredients may contribute to develop a palatable beverage. Nevertheless, it was showed that consumers, even though preferring the sensory properties of conventional juices to their functional counterparts, are inclined to prefer probiotic juices over the conventional one if the health benefits information is provided (Luckow & Delahunty, 2004).

Smoothies are one of the best examples of innovative vegetable-based fermented beverage. The manufacture of smoothies is based on the use of mixture of fruits and vegetables, often after removing seeds and peel, which are mainly processed to pulp or puree (Qian, 2006). In most of the cases, mixtures of fruits and vegetables are selected based on color, flavor, drinkable texture and, especially, to guarantee high concentration of nutrients with low energy content. Recently, a novel protocol for the manufacture of fermented red and green smoothies was established (Di Cagno, Minervini, Rizzello, De Angelis, & Gobbetti, 2011). White grape juice and *Aloe vera* extract were mixed with red (cherries, blackberries prunes, and tomatoes) or green (fennels, spinach, papaya, and kiwi) fruits and vegetables and were fermented by mixed autochthonous starters. A potential probiotic banana puree was obtained through fermentation by using k-carrageenan immobilized *L. acidophilus.* Cell immobilization may provide protective effects resulting in higher cell density, thus improving the fermentation efficiency (Tsen, Lin, & King, 2004, 2009).

The so-called vegetable milks might be also used as raw materials to develop yogurt-like products. Vegetable milks are mainly obtained from soy, cereals, and nuts, moreover others minor raw materials including hemp, sunflower, legumes (e.g., lupin and peanut seeds), and tubers (e.g., tiger nuts) are also used (Bernat, Cháfer, Chiralt, & González-Martínez, 2014a, 2014b; Bernat, Cháfer, Chiralt, Laparra, & González-Martínez, 2015; Beuchat & Nail, 1978; Wakil, Ayenuro, & Oyinlola, 2014). Yogurt-like products making process involves the following main steps (1) milk conditioning to the optimal growth temperature of the starters; (2) inoculation and incubation procedures; and(3) cooling at 4 °C (Bernat et al., 2014a). Proteins coagulation, and serum separation during storage, may cause problems

associated with physical stability. The addition of hydrocolloids, such as xanthan gum, modified starches, pectin, and cellulose derivatives, can be used to enhance physical stability. In situ production of oligosaccharides and exopolysaccharides by lactic acid bacteria positively affect the texture, since these compounds act as emulsifiers or stabilizers and increase viscosity and mouth feel (Coda, Lanera, Trani, Gobbetti, & Di Cagno, 2012). Probiotication was successfully reported for several vegetable milks. Prebiotic compounds (e.g., starch and fibers) may be added both to improve textural features and to support the probiotics survival (Bernat et al., 2014b, 2015; Mustafa et al., 2009; Santos, Libeck, & Schwan, 2014).

13.3.2 Fresh-Cut (Minimally Processed) Fruits and Vegetables

Fresh-cut (minimally processed) fruits and vegetables are obtained by trimming and/or peeling and/or cutting and packaging and are characterized by convenience and ability to maintain their freshness. Fermented pineapple slices by autochthonous lactic acid bacteria, also without any heat treatment, is an example of shelf-stable minimally processed fruit combining agreeable nutritional and sensory properties, and probiotic potential (Di Cagno, Cardinali, et al., 2010). Fermentation by autochthonous and selected lactic acid bacteria strains was demonstrated to guarantee an extended shelf-life for red and yellow peppers, which also maintained agreeable texture and sensory properties (Di Cagno et al., 2009). Fresh-cut apple wedges was developed by applying probiotic bacteria (*L. rhamnosus* GG) and prebiotics (oligofructose and inulin). The alginate coating, which was used as a carrier for prebiotic supplements, beneficially affected the stability of inherent polyphenols and it was able to retain apple volatiles slightly better than uncoated apple wedges (Rößle, Brunton, Gormley, Ross, & Butler, 2010). The alginate- and gellan-based edible coatings were successfully applied to support *Bifidobacterium lactis* viability on fresh-cut apple and papaya (Tapia et al., 2007). A protective effect against relevant foodborne pathogens was also reported. *L. rhamnosus* GG in fresh-cut apple wedges reduced the growth of *Listeria monocytogenes* (Alegre, Viñas, Usall, Anguera, & Abadias, 2011). *L. plantarum* B2 and *L. fermentum* PBCC11.5 showed a good ability to reduce the level of *Lis. monocytogenes* on artificially contaminated melons. Based on human trials, *L. paracasei* IMPC2.1 was recovered from fecal samples of the volunteers fed with artichoke heads carrying the strain, indicating that artichokes are suitable carriers for transporting bacterial cells into the gastrointestinal tract (Valerio et al., 2006).

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Chapter 14
Bioactive Compounds from Fermented Food Products

Maria Hayes and Marco García-Vaquero

14.1 Introduction

Fermentation is commonly used in the food and functional food industry and there are approximately 5000 varieties of fermented foods and beverages consumed worldwide (Michalak & Chojnacka, 2014; Tamang & Kailasapathy, 2010). Fermented food products include those derived from milk and dairy (yogurt, cheese and kefir), soy (natto and miso), fruits (wine), vegetables (sauerkraut) and fish and meats (FitzGerald & Murray, 2006). The secondary metabolites produced during fermentation processes range from antibiotics to peptides and are also referred to as bioactive compounds due to their biological activities which are numerous and range from the prevention of chronic diseases such as diabetes and cardiovascular disease to cancer prevention (Limón et al., 2015). These bioactive compounds improve the nutritional and bioactive profile of foods including meat (Olmedilla-Alonso, Jiménez-Colmenero, & Sández-Muniz, 2013), dairy (Raikos & Dassios, 2014), fish (Grienke, Silke, & Tasdemir, 2014), seaweeds (Shobharani, Halami, & Sachindra, 2013), microalgae (Barclay, Apt, & Dong, 2013), beverages (Marsh, Hill, Ross, & Cotter, 2014) and fruits and vegetables (Limón et al., 2015).

Fermentation processes may be divided into two systems: submerged fermentation and solid state fermentation. Submerged fermentation is based on cultivation of a microorganism in a liquid medium containing nutrients, whereas solid state fermentation consists of the microbial growth and product formation in the absence

M. Hayes (✉)
Food Biosciences, Teagasc Food Research Centre, Dublin 15, Ireland
e-mail: maria.hayes@teagasc.ie

M. García-Vaquero
Food Biosciences, Teagasc Food Research Centre, Dublin 15, Ireland

University College Dublin School of Agriculture and Food Science, Belfield, Dublin 4, Dublin, Ireland

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of water (Subramaniyam & Vimala, 2012). Solid state fermentation (SSF) uses solid substrates including bran and bagasse as well as nutrient-rich waste materials. During SSF, substrates are converted in a slow and steady manner to bioactive compounds. One advantage of this method over submerged fermentation processes is that it supports controlled release of nutrients. The method is suitable for use with fungi and microorganisms with less moisture requirements (Subramaniyam & Vimala, 2012). During submerged fermentation (SmF), the bioactive constituents are released into the growth/fermentation broth and the substrate is consumed rapidly. SmF is more suited to bacteria with high water activities but offers an advantage over SSF as the bioactive are easier to purify (Subramaniyam & Vimala, 2012). The fore-mentioned fermentation processes have been used traditionally to preserve foods and enhance the nutritional quality of foods. The type of microorganisms used during the fermentation process is a key parameter to its success. In order to be commercially viable, any fermentation process must be aware of the cellular functioning of the microorganism used. In milk, meat and vegetable fermentation processes, lactic acid bacteria (LAB) are traditionally used and it has long been thought that use of LAB including *Lactobacillus* genera and *Bacillus subtilis* can favour the production of bioactives (Limón et al., 2015; Song, Frias, Martinez-Villaluenga, Vidal-Valverde, & Gonzalez de Mejia, 2008; Torino et al., 2013). Furthermore, fermentation of dairy is dominated by LAB including *Leuconostoc*, lactobacilli and lactococci. Indeed, fermentation in colder climates involves mesophilic bacteria such as *Lactococcus* and *Leuconostoc* and thermophilic bacteria including *Lactobacillus* and *Streptococcus* are more dominant in warmer climates (Marsh et al., 2014).

Bioactive compounds including antimicrobial compounds produced during fermentation processes as secondary metabolites include hydroxy acids such as phenyl lactic acid, hydroxyphenyllactic acid, indoleacetic acid, alcohols such as phenylethyl alcohol as well as antimicrobial peptides. These bioactive compounds are known to play an important role in biopreservation of fermented food products including cheese, beer, sourdough, fermented vegetables and feed silage (Naz, Cretenet, & Vernoux, 2013). Furthermore, they can inhibit the growth of pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus* and *Enterococcus faecalis*. In addition to their antimicrobial properties, these metabolites can be aromatic and moreover, can influence the sensory and organoleptic characteristics of food products including the flavour and aroma profiles of fermented foods. Other bioactive compounds with health beneficial attributes can be produced during fermentation of food groups and include peptides which can impact and prevent diseases associated with metabolic syndrome (diabetes, CVD and obesity) (Abd Razak et al., 2015; Hayes, Ross, Fitzgerald, & Stanton, 2007; Hayes, Stanton, et al., 2007; Martins et al., 2011), phenolic compounds (Limón et al., 2015; Martins et al., 2011) and others.

In this chapter, we review the literature regarding traditional fermentation of food groups including marine (fish, seaweeds and marine microalgae), meat, dairy, fruits and vegetables and cereals for bioactive compounds with potential health benefits.

14.2 Marine Fermentation Processes and Production of Bioactive Compounds

14.2.1 Fish

Fermentation of fish was first introduced as a means of preservation, and fish sauces and pastes or condiments are staples of the diets of people from Southeast Asian, Scandinavian and within the Inuit cultures. The term "fermented fish products" refers to products from freshwater, marine finfish, shellfish and crustaceans that are processed with salt to cause fermentation and prevent putrefaction (Ruddle & Ishige, 2010). In general, fermented fish products are served mainly as a salty and umami condiment that assists in the consumption of large quantities of rice (Ruddle & Ishige, 2010). The prototypical product is shiokara which is produced in Japan (Ruddle & Ishige, 2010). Fermentation of fish meat can partially degrade the protein constituents which, as well as aiding absorption from the gut, may also influence its bioactive properties through the production of bioactive peptides.

Bioactive peptides refer to short sequences of amino acids that have biological activities that may influence human health above and beyond, basic, human nutrition (Kitts & Weiler, 2003; Mora & Hayes, 2015). Bioactive peptides derived from food proteins are inactive within the parent protein sequence and require release through hydrolysis with enzymes, proteolytic microbial strains or acids before becoming active (Erdmann, Cheung, & Schröder, 2008). They consist of between 2 and 20 amino acids. Peptides of marine origin have several bioactivities associated with them including cardio protective functions (Mora & Hayes, 2015) antioxidant (Najafian & Babji, 2012), mental health (Wilson, Hayes, & Carney, 2011) and general well-being functions (Ryan, Ross, Bolton, Fitzgerald, & Stanton, 2011). Table 14.1 shows a list of bioactive peptide containing commercial products and identified peptides that have been derived from fish hydrolysates and autolysates and other sources. Of these products, Peptide ACE3000 and Calpis are supported by the Japanese authorities (Thorkelsson, Slizyte, Gildberg, & Kristinsson, 2009). Furthermore, a thermolysin digest of katsuobushi was approved as a "food for special health use" (FOSHU) in Japan due to its blood pressure lowering properties (Vercruysse, Van Camp, & Smagghe, 2005). This product has not yet received a European Food Safety Authority (EFSA) approval for any health claim but does have approval for use as a novel food. The database BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) provides a useful resource when looking for peptides with different bioactivities and includes several fish derived peptides.

Table 14.1 Bioactive peptide containing commercial products derived from fish hydrolysates and autolysates and other sources

Company name	Commercial name of product	Source	Bioactivity/potential health claim	
Nippon Supplement, Japan	Katsuobushi oligopeptide	Bonito	ACE inhibitor	Fish sourced products
Senie Ekisu, Japan	Valtyron	Sardine muscle	ACE inhibitor	
Copalis, France	Protizen	White fish hydrolysate	Stress relief	
Copalis, France	Nutripeptide	White fish hydrolysate	IBS relief	
Copalis, France	Collagen HM	Hydrolysed fish collagen	Skin and cartilage regeneration	
Copalis, France	Prolastin	Hydrolysed fish skins	Ligament regeneration/ antioxidant	
Biothalassol	Fortidium liquamen	Molva molva	Lowers GI and stress relief	
Calpis, Japan.	Ameal S	Fermented milk product containing tripeptides	ACE inhibitor	Dairy sourced products
Valio, Finland	Evolus	Fermented milk product containing tripeptides	ACE inhibitor	
Davisco foods, USA	BioZate	Hydrolysed whey protein product	ACE inhibitor and antihypertensive	
DMV International, Holland	C12 peptide	Casein-derived peptide with 12 amino acids	ACE inhibitor and antihypertensive	

14.2.2 Seaweed

Seaweed fermentations for methane production were first studied in the 1970s and 1980s for energy supply purposes (Chynoweth, 2002; Japan Ocean Industries Association (JOIA), 1984). Fermented algal products dominate in the field of aquaculture. The use of seaweed as a biomass feedstock following fermentation is currently receiving renewed interest. Industrial consumption of edible crops, known as "first-generation biomass", has raised moral and ethical issues causing the industry to turn to "second-generation biomass" in the form of lignocellulose materials such as corn, wood and grass. However, processing of these materials can be costly due

to pre-treatment processes. Seaweeds are drawing attention as the "third-generation biomass", as their cultivation on non-arable land requires does not involve cropland used to produce food, and their low lignocellulose content lowers the effort and cost required for pre-treatment (Hwang, Lee, Kim, & Lee, 2011). Seaweeds are divided into three categories depending on their colour and pigmentation. These categories are the Chlorophyta (green seaweeds), Rhodophyta (red seaweeds) and Phaeophyta (brown seaweeds). Fermentation of seaweeds is a useful approach as it minimizes by-products and "wastes" produced following extraction of carbohydrates which are used in the development of technofunctional ingredients in foods and secondly it provides a useful method for utilising invasive algal species that can cause environmental problems (Michalak & Chojnacka, 2014). However, the cell walls of macroalgae are composed of rigid carbohydrates that can present some problems during the fermentation process. These major algal polysaccharides are known to be unfavourable substrates for fermentation (Uchida & Miyoshi, 2013). However, it was recently reported that seaweed could be used as a substrate for lactic acid and ethanol fermentation, provided that the algal tissue was saccharified with cellulase enzymes. In general, the use of both saccharification and fermentation are useful during seaweed fermentation processes. Saccharification utilizes polysaccharide degrading enzymes and fermentation involves the use of halophile, "salt-tolerant" microbial strains and the fermented product can be a liquid or a dried solid for use in the purification of other bioactive compounds including phlorotannins from the brown seaweeds and small molecules including polyphenols (Tierney, Croft, & Hayes, 2010; Wijesinghe & Jeon, 2012) and small, hydrolysed sugars (Michalak & Chojnacka, 2014). The fermentation process involves a change in colour of the algal hydrolysate due to chlorophyll degradation under acidic conditions and the observed microbial cell damage under the microscope (Uchida & Miyoshi, 2013). A number of fermented products produced from seaweeds are shown in Fig. 14.1 and include single-cell detritus (SCD) and algal silage.

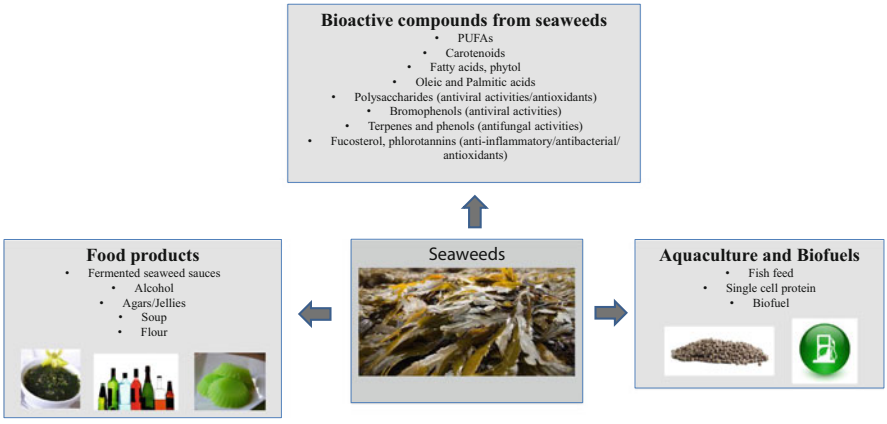

Fig. 14.1 Fermented products produced from seaweeds

A seaweed sauce product containing amino acids at the same level with soy and fish sauce products is prepared from *Nori*. Halophilic lactic acid bacteria originated from soy and fish sauces cannot grow in seaweed and suitable strains were isolated from the marine environment to develop the sauce product. Fermented seaweed is also used as silage in fish feed and could be used as biofuel and in alcoholic beverages.

Seaweed sauces produced using fermentation can be developed as foods. However, a major difficulty in developing seaweed sauces that have commercial value is that the supernatants of several fermented products produced from seaweeds have low amino acid content (Uchida & Miyoshi, 2013). The supernatant of fermented products prepared from seaweed, such as *U. pinnatifida* and *Ulva* sp. only contains 1–3 g N/100 mL of amino acid compounds and this is likely due to the low protein content observed in this species. However, fermented red seaweeds such as *Porphyra* sp. can contain high quantities of proteins that may exceed 50 %, depending on the season and location of harvest of this seaweed and amino acid-rich supernatants are obtained from this species (16.5 g N/100 mL) (Uchida & Miyoshi, 2013). Halophile LAB can be used in fermentations of seaweeds for product development.

14.2.3 Microalgae

Fermentation of the microalgal species *Chlorella* sp., *Tetraselmis* sp., *Pavlova lutheri, Chaetoceros* sp., and *Nannochloropsis* sp. as well as macroalgae was carried out previously using lactic acid bacteria (LAB) (Uchida & Miyoshi, 2010). Microalgal extracts are normally used in the cosmetic industry in skin care formulations acting where they can act as anti-aging, regenerating, antioxidant, anti-irritant, and also as sun protection agents in hair care products (Kim, Ravichandran, Khan, & Kim, 2008; Martins, Vieira, Gaspar, & Santos, 2014). Recently, a polysaccharide extract from microalgae *Parachlorella* and *Chlorella* sp. was patented for its cosmetics and cosmeceuticals applications and effects on human skin (Coragliotti, Franklin, Day, & Decker, 2015). Extracts from microalgae currently commercialized by different companies, the cosmetic claims and the microalgae from which these bioactive compounds are derived is shown in Table 14.2.

Polyunsaturated fatty acids (PUFAs), linolenic acid in particular (LA), play an important role preventing skin dehydration (Sivamani, Jagdeo, Elsner, & Maibach, 2015). Several vegetable oils rich in gamma-linolenic acid are used in cosmetics for damaged and healthy skin. Microalgal species that have a high content of PUFA and LA include *Spirulina platensis*, *Tetraselmis suecica*, *Porphyridium cruentum* and *Chaetoceros calcitrans* (Hirano et al., 2011). Also, microalgae rich in PUFA could be transformed in micro and nanoparticles with potential pharmaceutical applications (Lukowski, Juelich, Lindequist, & Mundt, 2006).

In addition, microalgae are rich in coloured proteins including phycoerythrin and phycocyanin produced by red and blue–green microalgae species. These colorants are found in cosmetics including eye shadows (Kim et al., 2008). Sporopollenin, a

Table 14.2 Commercially available microalgae based extracts

Name	Company	Chemical compound	Microalgae species	Cosmetic claims	References
Protulines®	Exsymol S.A.M., Monaco	Protein-rich extract	*Spirulina* sp.	Anti-aging, firming face and body	http://www.exsymol.com/eng/products/240-protulines. 10th February 2016
Photosomes®	AGI Dermatics, USA	Photolyase in liposome	*Anacystis nidulans*	UV protection of skin	Decome et al. (2005)
XCELL-30®	Greentech, France	Oligosaccharides-rich extract	*Halymenia durvillei*	Reduces the cell growth cycle of keratinocytes	http://www.greentech.fr/en/xcell-30-3/#comments. 9th February 2016
Dermochlorella DG®	Codif, France	Oligopeptides-rich extract	*Chlorella vulgaris*	Skin collagen synthesis, tissue regeneration and wrinkle reduction	Stolz and Obermayer (2005)
Alguronic Acid®	Algenist, USA	Polysaccharides and oil-rich extract		Anti-aging properties	
Alguar™	Frutarom, Israel	Sulphated polysaccharides	*Porphyridium* sp.	Anti-aging, wrinkle lifting and reduction properties	Coragliotti et al. (2015)
Pepha®-Tight	Pentapharm, Switzerland		*Nannochloropsis oculata*	Skin-tightening effects	
Pepha®-Ctive	Pentapharm, Switzerland		*Dunaliella salina*	Cell proliferation stimulation properties	
Blue Retinol™	Biotherm, France		*Dunaliella salina*	Stimulate the growth and proliferation of skin cells	Kim et al. (2008)

protein found in many microalgae species including *Dunaliella salina*, *Chlorella fusca* or *Scenedesmus* sp., scytonemin identified previously from *Nostoc punctiforme*, *Scytonema* sp. and *Chlorogloeopsis* sp. and mycosporine-like amino acids found in *Dunaliella tertiolecta*, *Chlorella minutissima* and *Chlorella sorokiniana* among others could have potential applications as natural sunscreens as these proteins and amino acid type compounds can protect against UV radiation (Borowitzka, 2013; Priyadarshani & Rath, 2012). Due to its antioxidant properties and UV irradiation protection properties, some cosmetics include in their formulations tocopherol which is produced in high quantities in the species *Euglena gracilis*, *Dunaliella tertiolecta* and *Tetraselmis suecica* and phenolic compounds (i.e., laurinterol and debromolaurinterol isolated from *Laurencia intermedia*) (Kim et al., 2008).

14.3 Meat Fermentation and Bioactive Components

Meat fermentation is a low-energy, acidulation method (due to lactic acid production, low water activity, salt and drying) that results in preservation and distinctive properties that include colour, microbiological safety, palatability, tenderness and other desirable attributes (Ockerman & Basu, 2014). Acidulation generally results from wild microorganisms/cultures which can lower pH. These microbial strains generally belong to the LAB. Spontaneously fermented meat products have a long tradition of production in certain regions of the world. The two major European producing and consuming countries for fermented meat products include Germany and France. Others include Spain, Italy, the Balkans, Hungary, Australia, the USA and Japan. Distinct production processes and artisanal microflora lead to a great diversity and variety of fermented meat products (Čaklovica et al., 2005; Čolo et al., 2015). Traditional fermented meat products include Italian salami, Spanish salchichon and chorizo, Icelandic Slátur (blood sausage), Irish pig-blood derived black pudding (blood sausage), beef sticks, pepperoni and others including Bosnian sudžuk—a dry fermented beef sausage produced in a rural household near the town of Visoko in central Bosnia and Herzegovina (Čolo et al., 2015) and Salami Milano in Italy (Talon et al., 2007).

Several ACE-I inhibitory peptides have been isolated from muscle proteins to date and have been reviewed by a number of authors (Ahhmed & Muguruma, 2010; Di Bernardini et al., 2012; Vercruysse et al., 2005). Proteolytic protein degradation occurs in fermented meat products and is known to release bioactive peptides during the process. Previously, angiotensin-I-converting enzyme inhibitory peptides and antioxidant peptides were identified from cured ham and fermented sausages (Ferranti et al., 2014). identified the DPPH radical scavenging activity and ACE-I inhibitory activity of protein extracts from Petrovac sausage that increased with ripening of the product. In addition, Sentandreu and Toldrá identified seven bioactive ACE-I inhibitory peptides from porcine skeletal muscle treated with dipeptidyl peptidase previously (Sentandreu & Toldrá, 2007). These included the peptides RP, KA, AA, GP, AR, GR and RR. Furthermore, Spanish dry-cured ham as a natural

source of antioxidant and ACE-I inhibitory peptides has been extensively investigated in recent years (Escudero et al., 2013; Escudero, Mora, & Toldrá, 2014; Gallego, Mora, Aristoy, & Toldrá, 2015). DPPH activities ranging from 39 to 92 % and superoxide ion extinguishing ability ranging from 41 to 50.27 % were identified for peptide extracts isolated from Spanish dry-cured ham (Escudero, Aristoy, Nishimura, Arihara, & Toldrá, 2012; Stadnik & Keska, 2015). In addition, Ferranti et al. (2014) identified over 170 bioactive peptides from the bovine muscle proteins of the dry-cured meat product Bresaola.

Dry fermented sausages have three phases for production including formulation, fermentation and ripening/drying (Čolo et al., 2015; Sanz, Hernández, Ferrús, & Hernández, 1998). The ripening technique has an effect on the LAB composition, which develops during the fermentation process. Among the LAB, *Lactobacillus* species including *Lactobacillus sakei*, *L. curvatus* and *L. plantarum* as well as *Leuconostoc*, *Weissella*, *Enterococcus*, *Pediococcus* and *Lactococcus* are the genera most widely described in fermented sausages (Di Cagno et al., 2008). Lactobacilli are often present in the early stages of sausage fermentation, whereas there is no pattern of distribution of the other LAB groups during fermentation (Čolo et al., 2015; Urso, Comi, & Cocolin, 2006). Staphylococci were isolated previously from black pudding with *S. saprophyticus* and *S. carnosus* the most prevalent isolates. The detection of enterotoxin-encoding genes and resistance in staphylococcal isolates from Brazilian black pudding indicated that this fermented food may represent a potential health risk, since staphylococci present in food could cause foodborne diseases or be a possible route for the transfer of antimicrobial resistance to humans (De Moura et al., 2012).

Arihara first described the use of probiotic bacteria in fermented meat products. Probiotics including bifidobacteria and LAB can provide organoleptic and nutritional advantages as well as health benefits to the fermented meat products. Several meat products containing probiotics with claims for health benefits have been commercialized. Salami containing three intestinal LAB (*Lactobacillus acidophilus*, *Lactobacillus casei* and *Bifidobacterium* spp.) was produced by a German company in 1998 and a meat spread containing an intestinal LAB (*Lactobacillus rhamnosus* FERM P-15120) was produced by a Japanese company. Fermented sausages are suitable for the incorporation of probiotic bacteria since mild or no heat treatment is usually required by dry fermented meat products, thus providing the suitable conditions required for the survival of probiotics. The sausage has to be designed in such a way as to keep the number and viability of probiotic strain in the optimum range. Thus, reduction in pH (e.g. <5.0), extended ripening (e.g. >1 month), and dry or excessive heating has to be avoided if the beneficial effects of probiotic are to be harvested.

In the meat sector, meat cultures are generally added to fermented meat products with the goal of extending shelf life. However, there are no documented studies concerning the isolation of bioactive peptides from fermented meat products developed with probiotic strains to date. By-product fermentation from meat processing has also been used to generate bioactive compounds. Goat placenta has long been used in Chinese medicine and is recognized as rich in biological and therapeutic

components (Chakraborty & Bhattacharyya, 2005). The preparation of goat placenta peptides using fermentation was assessed recently by Hou et al. (2014). They identified that the DPPH scavenging activity of goat placenta peptides reached 85.43 % at a concentration 1.4 μg/mL. The 50 % inhibition concentration (IC_{50}) value of peptides was calculated by nonlinear regression to be 0.84 μg/mL.

14.4 Cereal-Derived Fermented Products

Traditional fermented cereal beverages include Boza which is produced in Bulgaria and Turkey from grains including barley, oats, rye, millet, wheat or rice (Akpinar-Bayizit & Lutfiye, 2010; Marsh et al., 2014). The grains are boiled and filtered and then a carbohydrate source is added and the mixture is left to ferment naturally. The microbial population responsible for fermentation is wild, but it is thought to be produced by any of the following strains: *S. cerevisiae*, *Leuconostoc mesenteroides* and *Lactobacillus confusus* (Zorba, Hancioglu, Genc, Karapinar, & Ova, 2003). In Russia, Kvass is produced from rye grain and is a popular alcoholic drink in this country (Jargin, 2009). Other fermented cereal products include Ogi, a fermented cereal porridge consumed in Nigeria, Amazake a sweet fermented rice beverage consumed as an alternative to sake in Japan and Pozol which is produced in south-eastern Mexico from maize grains (Ben Omar & Ampe, 2000).

There is a strong association between fermented cereal products, the microbial content and improvement of gastrointestinal health and evidence exists that these fermented products provide beneficial health effects through probiotic mechanisms and via the production of secondary metabolites and breakdown products produced during the fermentation process (Marsh et al., 2014). In addition, fermented cereals often have a high mineral content and are low in fat, but they lack essential amino acids (Marsh et al., 2014). However, they also contain fibre, vitamins minerals, flavonoids and phenolic compounds that can provide antioxidative and antiinflammatory benefits to the consumer (Wang et al., 2014). Organic acids lower the pH of fermented cereal products and may also confer health benefits.

14.5 Dairy Fermented Products

Milk contains a number of bioactive components that can guard against illness and disease and impart health benefits to the consumer that go above and beyond basic human nutrition (Park, 2009). These bioactive components include bioactive peptides, immunoglobulins, antimicrobial proteins and peptides, oligosaccharides, lipids and minor components that can benefit the consumer. Bioactive peptides from dairy products are reported to possess antimicrobial, antioxidative, immunomodulatory, ACE-I inhibitory and renin inhibitory bioactivities (Park, 2009). The major

proteins in milk include caseins (αs1, αs2, β and k), β-lactoglobulin, α-lactalbumin, immunoglobulins, glycomacropeptide, lactoferrin, lactoperoxidase, lysozyme and serum albumin.

Milk is rich in lipids and contains over 400 fatty acids with different chemical compositions including conjugated linoleic acid (CLA). Milk fat is the richest source of CLA and reports have indicated levels ranging from 2.0 to 53.7 mg/g. CLA refers to a collection of positional and geometric isomers of cis-9, cis-12-octadecadienoic acid (C18:2) with a conjugated double bond system. The most common CLA type 9-cis, 11-trans also known as rumenic acid (Collomb, Schmid, Sieber, Wechsler, & Ryhanen, 2006) is found in milk fat. Well-known health effects attributed to CLA include anticarcinogenic, antiatherogenic and antidiabetic effects (Park, Storkson, Albright, Liu, & Pariza, 1999).

Other bioactive components present in fermented and non-fermented dairy products include the immunoglobulins (IgA, IgG and IgM) that are found in the milk of all lactating species. α-Lactalbumin is a precursor of serotonin and glutathione and has been reported to prevent stress (Korhonen & Pihlanto, 2006).

As already stated, bioactive peptides are sequences of amino acids that range between 2 and 30 amino acids in length and provide a hormone-like beneficial effect to the humans (Hayes, Ross, et al., 2007; Hayes, Stanton, et al., 2007). Bioactive peptides were first identified from colostrum and were first assigned bioactivities in 1950 when Mellander reported that ingestion of casein-derived phosphorylated peptides led to enhanced vitamin D-independent calcification in rachitic infants. They are produced by fermentation, hydrolysis and high pressure treatment, but they are also present naturally in a number of fermented food products including cheese, yoghurts and fermented milks. For example, Calpis is popular fermented milk drink in Japan which is beneficial for heart health due to the presence of various bioactive peptides. The bioactive peptides Isoleucine–Proline–Proline (IPP) and Valine–Proline–Proline (VPP) found in Calpis can inhibit the enzyme angiotensin-converting enzyme I (ACE-I; EC 3.4.15.1) which is involved in the renin–angiotensin–aldosterone system (RAAS). IPP and VPP can inhibit this enzyme and prevent the formation of Angiotensin II, a vasoconstrictor, and the subsequent constriction of blood vessels and elevated blood pressure in the consumer (Hayes, Ross, et al., 2007; Hayes, Stanton, et al., 2007). Other ACE-I inhibitors are found in different varieties of cheeses and yoghurt products as shown in Table 14.3. Several dairy starter cultures are proteolytic and thus can generate bioactive peptides from dairy proteins. Examples of proteolytic dairy starter cultures include *Lactobacillus helveticus*, *Lactobacillus delbrueckii, Lactobacillus plantarum*, *Lactobacillus rhamnosus, Lactobacillus* acidophilus and *Lactococcus lactic and Streptococcus thermophilus* (Hajirostamloo, 2010). In order to produce bioactive peptides in dairy products, fermenting with a highly proteolytic strain is necessary. The next section discusses microbial strain selection.

Table 14.3 ACE-I inhibitory peptides found in traditional fermented dairy products

Fermented food	Protein fragment	Bioactivity	References
Cheddar cheese	αs1- and β casein fragments	ACE-I inhibition	Singh, Fox, and Healy (1997)
Mozzarella cheese	β casein f(58-72)	ACE-I inhibition	Smacchi and Gobbetti (1998)
Gorgonzola cheese	β casein f(58-72)	ACE-I inhibition	Smacchi and Gobbetti (1998)
Gouda cheese	αs1-casein f(1-9)	ACE-I inhibition	Saito, Nakamura, Kitazawa, Kawai, and Itoh (2000)
	β-casein f(60-68)	ACE-I inhibition	Saito et al. (2000)
Manchego from sheep milk	αs1, αs2 and β caseins	ACE-I inhibition	Gómez-Ruiz, Ramos, and Recio (2002)
Sour milk	β casein f(74-76) and f(84-86)	ACE-I inhibition	Nakamura et al. (1995)
Dahi	Ser-Lys-Val-Tyr-Pro	ACE-I inhibition	Ashar and Chand (2004)

14.6 Microbial Strain Selection

Probiotics are defined by the world health organization (WHO) as "live microorganisms, which when administered in adequate amounts confer a health benefit to the host". Lactic acid bacteria (LAB) including lactobacilli and bifidobacteria are physiologically suited to the gut environment and are widely used probiotics. Indeed, the end products of LAB metabolism often include a number of vitamins such as folate, vitamin B12, riboflavin and vitamin K and as such products are often produced during dairy fermentations prior to consumption, they undoubtedly impart additional health benefits (Barrett et al., 2005). However, there are several technical challenges associated with the stabilization and indeed the European Food Safety Authority (EFSA) have not, to date, granted a health claim to any probiotic food product on the market. Strict criteria exists surrounding the use of the term "probiotic" in Europe (Guarner et al., 2011) and evidence must be provided by a company linking the presence of beneficial probiotic bacteria to a physiological benefit. Furthermore, probiotics must be present at 10^9 colony forming units (CFU) per ml to be effective (Marsh et al., 2014).

Rational starter culture selection is necessary to ensure reproducibility of any fermented food product and sourcing microbes from traditional fermented foods is a good place to start as these microbes are clearly surviving in an environment that suits their growth requirements (Marsh et al., 2014). Furthermore, microbes isolated from traditional fermented products have a history of safe use and produce a product that meets the consumers' taste, flavour and aroma approval. The first inventory of microorganisms with a documented history of use in food was compiled in 2001 by the International Dairy Federation (IDF) and the European Food and Feed Cultures Association (EFFCA). This was updated in 2012 and now covers a wide

range of food applications (including dairy, fish, meat, beverages and vinegar) and features a reviewed taxonomy of microorganisms (Bourdichon et al., 2012).

Molecular-based microbial characterization of traditional fermented food products is necessary in order to bridge the gap between associated health claims and reliable production of health beneficial fermented foods. Techniques including DGGE can assess 1–2 % of a microbial population and has been used (Muyzer, de Waal, & Uitterlinden, 1993). However, high-throughput, sequencing-based microbial analysis coupled with metabolomics and bioinformatics can provide a more accurate picture of the microbial population of a fermented food product (Marsh et al., 2014; Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013). Furthermore, sensory challenges that must be overcome include the production of sour, acidic flavours and the use of sensory panels is necessary to produce fermented products that consumers will want to purchase. To this end, careful microbial and substrate selection is necessary and controlled fermentation processes that utilize chemistry methods including chromatography (RP-HPLC, HPLC and GC) and mass spectrometry will help to overcome this challenge. Bioassays for antioxidant activities and health benefits should also be employed during the characterization procedure of any fermented product.

14.7 Conclusion

As demonstrated, food-grade microbes are useful cell factories for producing and delivering valuable secondary metabolites including bioactive peptides, vitamins, fatty acids and polyphenols that can positively impact human health and nutrition. For the production of fermented foods rich in bioactive compounds, microbial strain selection is an important parameter and traditional fermented food products provide a useful reservoir to "mine" such microbes using biotechnological methods.

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Chapter 15
Innovations in Packaging of Fermented Food Products

Begonya Marcos, Carmen Bueno-Ferrer, and Avelina Fernández

15.1 Introduction

Food packaging is designed to contain and protect foods, to provide required information about the food and to make food handling convenient from distribution to consumer's table. The primary functions of packaging are to achieve preservation and the safe delivery of food products until consumption (Han, 2014). Food packaging technology is continuously evolving in response to growing challenges from a modern society (Realini & Marcos, 2014). Major current and future challenges to fast-moving consumer goods packaging include legislation, global markets, longer shelf life, convenience, safer and healthier food, environmental concerns, authenticity and food waste (Kerry, 2014). This background offers a unique opportunity to the packaging industry to offer innovative solutions to address the changing demands of the food industry and consumers as well as the increasing regulatory and legal requirements (Han, 2014). This chapter will present innovations and trends applied to the packaging of fermented food products: optimisation of barrier properties, modified atmosphere packaging, adaptation of packaging to non-thermal preservation technologies, active packaging and sustainable design.

B. Marcos (✉)
IRTA, Food Technology, Finca Camps i Armet s/n, Monells 17121, Spain
e-mail: begonya.marcos@irta.cat

C. Bueno-Ferrer
School of Food Science and Environmental Health, Dublin Institute of Technology, Cathal Brugha Street, Dublin 1, Ireland

A. Fernández
Instituto de Física Corpuscular (Consejo Superior de Investigaciones Científicas-Universitat de València) Parc Científic UV, Paterna 46980, Valencia, Spain

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15.2 Barrier Technologies for Fermented Food Packaging

For any given product, it is necessary to understand the most essential material requirements to achieve the targeted environment inside the package and the optimal food quality (Jansson et al., 2002). Therefore, an adequate selection of the barrier packaging material can slow down the rate of food quality deterioration, thus extending the shelf life of the fermented food products (Steinka, Morawska, et al., 2006). Vapours and gases produced or consumed during metabolic processes in fermented foods may require more complex packaging barrier than other products, so in these cases it becomes crucial to find the right balance between oxygen barrier and carbon dioxide permeability. High gas barrier films are generally represented as linear chains with aromatic or polar groups in high proportion and with high molecular weight. Barrier layers made of polymers like PA (polyamide), PET (polyethylene terephthalate) or PVC (polyvinyl chloride) with materials like EVOH (ethylene vinyl alcohol), PVOH (polyvinyl alcohol) or PVDC (polyvinylidene) embedded in the multilayered structure are frequently used as barrier systems in food packaging. However, the use of multilayered films including a barrier layer like EVOH might not be desirable in terms of material recyclability. New developments in transparent, eco-friendly and barrier films include the incorporation of silica oxide (SiOx) coating to the package, among other alternatives (Lee, 2010).

Manufacturers of fermented dairy foods, such as yoghurt, face difficulty in maintaining the viability of bacteria over the shelf life of the products due to several factors including product acidity, pH, storage temperature and oxygen levels. High barrier to O_2 is needed as probiotic including lactic acid bacteria are predominantly anaerobic, and an increase in oxygen inside the package could be detrimental to their survival and growth. Miller, Nguyen, Rooney, and Kailasapathy (2002) investigated the effect of different packaging materials on the amount and distribution of oxygen dissolved in stirred-type probiotic yoghurt. High impact polystyrene (HIPS) was used as a control and a high gas barrier material consisting of a laminate made of HIPS and PE (polyethylene) as moisture barriers and intermediate layer of EVOH providing gas barrier was used for comparison. The use of packaging materials with enhanced barrier properties was shown to reduce the dissolved oxygen concentration in yoghurt during storage, leading to a low and stable oxygen concentration below 10 ppm after 42 days at 4 °C, while samples packaged in HIPS reached values of 50 ppm at the end of shelf life. Jansson et al. (2002) studied the content of O_2 and CO_2 in fermented milk packed with high density polyethylene (HDPE) as control film and two different LDPE (low density polyethylene) multilayer films containing EVOH (LDPE/EVOH32/LDPE, LDPE/EVOH44/LDPE) and an aliphatic polyketone (LDPE/PK/LDPE). EVOH32 pouches showed a lower CO_2 concentration and higher O_2 concentration after 8 days storage at 8 °C. The authors highlighted that CO_2:O_2 content ratio can be varied over a wide range by varying crystallinity and polarity materials.

Shelf-life extension of cheese with short maturation times is another interesting area of investigation in terms of improving properties of packaging materials. Changing the light transmission characteristics of food packaging materials by colouring the materials may be a suitable way of reducing photo-oxidative quality changes in cheese. Mortensen, Sørensen, and Stapelfeldt (2002) proved that oriented polyamide (OPA)/PE packaging in black laminates provided the best barrier protection for Havarti cheese, followed by OPA/PE white laminates.

An increasing concern in environmental issues has recently brought a growing number of bio-based formulations in the field of food packaging as will be discussed later. Nevertheless, one of the main disadvantages of these environment friendly materials is their poor barrier properties, thus such properties will have to be improved prior to their application in fermented food products packaging. Peelman et al. (2014) evaluated the influence of the use of bio-based packaging material on the quality and shelf life of grated cheese packaged under 100 % CO_2 atmosphere in PET-PE pouches as a conventional material and in (1) cellulose-starch and (2) metallised cellulose-starch pouches as an alternative bio-based packaging material with low oxygen and moisture permeability. The use of barrier improved bio-based packaging materials contributed to improve the sensorial perception, quality parameters and microbial counts of grated cheese.

Meat and meat products are highly susceptible to lipid oxidation and microbial spoilage, which can lead to the development of rancid or off-flavours. Meat products are commercially packed using vacuum or modified atmosphere conditions in high barrier plastic multilayer films. Several researchers investigated the possibility of extending shelf life of traditional dry fermented sausage (Petrovac sausage) applying a bio-based collagen-chitosan based coating to the fermented sausage as a substitute of traditional collagen coatings (Krkić et al., 2013; Krkić, Lazić, Petrović, Gvozdenović, & Pejić, 2012; Krkić, Lazić, Savatić, et al., 2012). The chitosan-collagen coating showed lower O_2 permeability and was able to slow down moisture loss and lipid oxidation, resulting in better sensory properties. Working with the same type of dry fermented sausage, Ščetar, Kovačić, Kurek, and Galić (2013) performed a different approach, by using a combination of different laminates as external packaging (LLDPE/EVOH/PET and PVDC/Polyester/PE) together with vacuum or modified atmosphere packaging (MAP) (100 % N_2) at three different storage temperatures. The PE-LLD/EVAL/PET laminate, which had the lowest O_2 permeability, scored the best on sensory attributes of dry fermented sausage during storage (upto 120 days) at 4 °C packaged under 100 % N_2 atmosphere.

Pressure built up in packaged fermented vegetable products may result in volume expansion and leakage problems. Therefore, optimal packaging for red pepper paste needs to meet the roles of alleviating volume expansion and preserving product quality. In the study carried out by Lee, Hwang, Choi, and Lee (2003), fermented red pepper paste was packaged under air and MAP of 30 % CO_2/70 % N_2 and 100 % CO_2. The MAP conditions were applied with a high gas barrier film (Nylon/EVOH/LLDPE) and with a gas permeable film (coextruded multilayered Nylon). Difference in internal atmosphere among the packages existed only in the initial period of storage. The balance between CO_2 production from the red pepper paste and permeation through the film with high O_2

and CO_2 permeability reached similar gas compositions after 50 days for all the packages. Shin, Cheigh, and Lee (2002) studied the effects of CO_2 absorption on the packaging material used to pack kimchi, a fermented vegetable dish made of salted Chinese cabbage with spices. Barrier properties were improved by fabricating plastic sheets made of PS (polystyrene) or PE and incorporated with Na_2CO_3-zeolite powder (20 w/w%), which were uniformly distributed in the polymer matrix. When sodium carbonate was used along with zeolite, the CO_2 absorption of the latter was helped due to the reaction of the former with water, alleviating pressure build-up and volume expansion of kimchi packages while maintaining a low stabilised CO_2 partial pressure.

15.3 Modified Atmosphere of Fermented Food Products

15.3.1 MAP Principles

Food spoilage is mainly due to moisture loss or uptake, fat oxidation and microbial growth. Storage of foods in a modified atmosphere can maintain quality and extend product shelf life. The function of MAP is to exclude oxygen and moisture from the packaged food and thereby retard growth of spoilage microorganisms, reduce oxidative rancidity, and maintain texture and colour of various food products (Aidlin, Arch, et al., 1997). Selection of the most appropriate packaging materials is essential to maintain the quality and safety of MAP foods. MAP requires the use of high barrier materials such as the ones described in the previous section (see Sect. 15.2).

The gases used in MAP of fermented food products are carbon dioxide and nitrogen. CO_2 is used in MAP of foods for its bacteriostatic and fungistatic properties. It is particularly effective against moulds and Gram-negative, aerobic spoilage bacteria such as Pseudomonas sp., but it is much less effective in controlling yeasts or lactic acid bacteria (Robertson, 2013). CO_2 dissolves readily in water to produce carbonic acid resulting in a pH reduction. As with all gases, the solubility of CO_2 increases with decreasing temperature and therefore the antimicrobial activity of CO_2 is higher at low temperatures (Robertson, 2013). The high solubility of CO_2 in high moisture/high fat foods can result in package collapse due to the reduction of headspace volume. Optimum levels of CO_2 to control the bacterial and mould growth are in the range of 20–30 % (Mullan & McDowell, 2003). N_2 is a relatively unreactive gas which is commonly used to displace air, particularly oxygen, from the package. Oxygen removal results in growth inhibition of aerobic spoilage microorganisms. N_2 is also used to balance gas pressure inside packs in order to prevent the collapse of packs containing high moisture and high fat food products (Mullan & McDowell, 2003). Noble or inert gases such as argon are also commercially used as filler gases; however, the literature on their application and benefits is still limited (Mullan & McDowell, 2003).

The proper combination of food, gas mixture and package material will result in extension of shelf life and improved food quality.

15.3.2 MAP of Fermented Food Products

Deterioration of fermented meat products during storage is mainly due to discoloration, biochemical changes, fat oxidation and microbial spoilage (Lawrie & Ledward, 2006). Nitrosomyoglobin (NOMb), the pigment of cured meats, is stable in the absence of O_2, but its oxidation to metmyoglobin is very fast in the presence of O_2. The rate of NOMb oxidation increases directly with increasing O_2 tension and is accelerated by the action of light (Robertson, 2013). The key aspect to improve the quality of packaged fermented meat products is to reduce the presence of oxygen which can be achieved by means of vacuum packaging and MAP (20–30 % carbon dioxide and 80–70 % nitrogen). These atmospheres reduce discolouration and fat oxidation and inhibit the growth of microorganisms. Esturk and Ayhan (2009) reported improved quality of sliced salami packed in the absence of O_2 (100 N_2, 50 % CO_2/50%N_2). Other authors have reported a reduced production of biogenic amines (putrescine) in fermented sausages (a_w=0.915) packed with 70 % CO_2/30%N_2 (Tabanelli, Montanari, Grazia, Lanciotti, & Gardini, 2013). Meat products are commonly packed in MAP using semi-rigid and rigid trays and the gas replacement is carried out by removing air present in the package using vacuum followed by gas flushing. To better maintain the integrity of the package, total pressure inside the package is maintained slightly below 1 atm (Toldrá, Gavara, & Lagarón, 2004). In general, MAP is not considered as an ideal choice for long storage of mould-ripened sausages. Packaging in high barrier materials prevents moisture to evaporate from the surface, resulting in mould loosening and giving the product a bad appearance (Incze, 2004).

Microbial growth and rancidity are the primary causes of quality deterioration in dairy products. The type of spoilage largely depends on the characteristics of the particular product. The main limitation for the shelf life of yogurt and fermented milk is the spoilage by bacteria, moulds and yeasts that grow at refrigeration temperatures. In addition, syneresis and oxidation are also considered as the main limiting factors for yogurt shelf life (Entrup, 2005). N_2 flushing of package headspace has proved to be able to extend the shelf life of yogurt. On the other hand, CO_2 addition through modified atmosphere packaging or direct injection as a cost-effective shelf-life extension strategy is used commercially worldwide (Hotchkiss, Werner, & Lee, 2006). Liquefied or compressed CO_2 gas can be incorporated directly into a flowing stream of product which has advantages over conventional MAP in that no headspace is required and the amount of dissolved CO_2 can be carefully controlled (Hotchkiss et al., 2006). Wright, Ogden, and Eggett (2003) determined that the threshold of carbonation in yogurt to extend shelf life without changing sensory properties was around 5.97 mM. Yogurt produced under regular conditions has a shelf life of 10–14 days at 4–6 °C that can be extended to 22–25 days when packed in MAP condition of 0–30 % CO_2/100–70 % N_2.

Hard cheeses with relatively low water activity are normally affected by the growth of moulds while products with high water activity such as soft cheeses are more susceptible to fermentation and rancidity. MAP was proven to be useful in

prolonging the shelf life of cheese samples in terms of microbiological and sensorial aspects. The selection of gas mixtures depends on cheese type, cheese manufacturing conditions, initial microbial load, packaging materials and storage conditions, as well as post-processing handling (Khoshgozaran, Azizi, & Bagheripoor-Fallah, 2012). MAP is used particularly for cheeses that are more prone to deteriorative changes such as portioned and sliced hard with a large surface area exposed to light and O_2 (Robertson, 2013). Hard and semi-hard cheeses, such as cheddar, are commonly packed in 100 % CO_2 or N_2/CO_2 mixtures in order to prevent mould growth (Hotchkiss et al., 2006). Favati, Galgano, and Pace (2007) reported that Provolone cheese packed with CO_2 and N_2 (30:70) extended its shelf life to more than 9 months at 8 °C slowing down the proteolytic and lipolytic phenomena during cheese ripening. Other authors also observed inhibition of *E. coli* and total viable counts in Graviera cheese packed in MAP (Arvanitoyannis, Kargak, & Hadjichristodoulou, 2011). MAP is also used for sliced and grated cheeses to inhibit mould growth and to facilitate separation of the portions. Soft cheeses are also packaged in atmospheres with increased carbon dioxide and low oxygen levels to inhibit bacterial growth and rancidity. However, for soft cheeses as the water content is higher, the concentration of CO_2 has to be limited to 40 % to avoid collapse of the package as well as formation of undesirable flavours during storage (Zhao, 2005).

The most common forms of deterioration of bakery products are microbiological spoilage and moisture loss or gain during storage. MAP is the most common packaging technology used to extend the shelf life of bakery products due to its high air content and fragile structure which cannot be vacuum-packed. A range of gas mixtures has been used to extend the shelf life of bakery products ranging from 100 % CO_2 to 50 % CO_2/50 % N_2 (Robertson, 2013). N_2 acts as a filling gas while CO_2 is used for its bacteriostatic and fungistatic action (García Iglesias, Gago Cabezas, & Fernández Nuevo, 2006). Extensions of 3 weeks to 3 months at room temperature are achievable using appropriate mixtures of CO_2 and N_2 (Smith, Daifas, El-Khoury, Koukoutsis, & El-Khoury, 2004). In this sense, Degirmencioglu et al. (2011) reported that sliced bread samples packed in MAP (100 % N_2, 70 % N_2/30 % CO_2, 50 % N_2/50 %CO_2, 30 % N_2/70 %CO_2 and 100 % CO_2) showed no growth of moulds after 21 days of storage at 20 °C and 60 % RH, being MAP with 100 % CO_2 the most effective treatment for the inhibition of bacteria. Other authors have reported extensions of shelf life of 117 and 158 % of sliced wheat bread packed in CO_2:N_2 (50:50) stored at 20–25 °C and 15–20 °C, respectively (Rodríguez, Medina, & Jordano, 2000).

15.4 Effect of Non-thermal Food Processing Technologies on Packaging Materials

Non-thermal processing technologies comprise a number of novel techniques used to preserve and enhance the quality and safety of food by a less aggressive approach than traditional thermal preservation methods. These new food processing technologies are usually non-thermal, resulting in fresh like taste, flavour and reduced

nutrient losses than traditional heat-based processing technologies. Most of these technologies are applied directly on the packaged food product in order to prevent post-processing contamination. However, to date little attention has been paid to the influence of these non-thermal treatments on food packaging materials with regard to mechanical, structural, thermal and barrier properties. Therefore, the assessment of packaging properties, mainly barrier properties, when subjected to different food processing conditions gains a particular relevance.

Among these technologies, high pressure processing (HPP), pulsed electric fields (PEF) and radiation are some of the main techniques currently being used at industrial scale. Others like ionising radiation, plasma or ozone treatment are presently in its infancy, and many other techniques such as magnetic fields, ultrasound, pulsed light, high voltage arc discharge, dense phase carbon dioxide, UV radiation, electron beam or pulsed X-ray represent a noticeable but minor alternative processing methods (Morris, Brody, & Wicker, 2007).

Foods can be irradiated after packaging by gamma radiation, e-beam or intense light. Irradiation can inactivate microorganisms and change physiological responses of foods; however, it can also change the chemical structure of polymeric packaging materials (Min & Zhang, 2007). For this reason, packaging materials used for irradiation should be chemically stable under the radiation dose to prevent polymer degradation and low molecular weight hydrocarbons and halogenated polymers formation which can migrate into foods. Radiolysis products (RPs) formed upon irradiation of a polymer or adjuvant could migrate into food and can affect odour, taste and safety of the irradiated food. Radiation does not generally affect all properties of a polymer to the same degree. According to Mrkić, Galić, and Ivanković (2007), barrier properties of some monofilms (PE-HD, PE-LD, PS, BOPP) are not significantly changed by irradiation (Rojas Gante & Pascat, 1990). As in the case of laminates, barrier properties either decrease, as in the case of BOPP/CPP and PET/PVDC/PE (Kim-Kang & Gilbert, 1991; Mizani, Sheikh, Ebrahimi, Gerami, & Tavakoli, 2009), or are not significantly affected, for example PA/PVDC/EVAC, PET/PE/EVAL/PE and PET/PET/PE-LLD (Deschênes et al., 1995; Mizani et al., 2009; Riganakos, Koller, Ehlermann, Bauer, & Kontominas, 1999), with applied radiation doses.

HPP is used to reduce food spoilage and pathogenic microorganisms from solid and liquid food products, extending the shelf life with minimal impact of the quality and nutritional value of food. High pressure-induced damages in polymeric packaging materials can be split into two categories: direct and indirect effects. Direct effects are caused directly by the high pressure treatment while the indirect effects are caused by the compression of other substances in the package. A direct effect of high pressure treatment on polymers can lead to changes in crystallinity. Delamination and other changes may also occur in multilayer packaging which affects the overall functionality of the whole package. Multilayer systems that include inorganic layers frequently see direct damages to the inorganic layers and delamination at the interface between inorganic and polymeric layers with both of these problems caused by the discontinuity of the mechanical properties across the interface. Indirect effects of high pressure treatment on polymeric packaging materials are primarily caused by compressed gases which will initially cause thermal

effects ranging from localised increase of crystallinity to more serious problems such as melting of the sealing layer (Fleckenstein, Sterr, & Langowski, 2014).

The effect of high pressure processing (400 MPa for 30 min, at 20 or 60 °C) on mechanical and thermal properties of four complex packaging materials (PE/EVOH/PE, metallised PET/PE, PET/PE, PP-SiOx) was studied by Galotto et al. (2008). Delamination and wrinkling were observed as a general consequence of the HP processing of multilayer polymeric systems without influencing mechanical properties of PE containing laminates. SiOx broke down after the HP treatment, thus causing significant modifications in PP-SiOx laminates. Other authors investigated the effects of HP pasteurisation (25 °C) and HP sterilisation (90 °C) on the cause of onset delamination of bilayer films (PP/PA, PP/OPA, PP/PET) using water and solid carrots as food stimulants (Fraldi et al., 2014). Each of the three bilayer films that were tested was able to withstand HPP at 25 °C without showing any evident mechanical failures. Moreover, there was no delamination after high pressure sterilisation at 90 °C, even at 700 MPa for PP/PA (Fig. 15.1c) and was barely present for PP/OPA (Fig. 15.1d) for both food simulants. However, the PP/PET pouches showed signs of localised delamination after high pressure sterilisation over the entire investigated pressures range (Fig. 15.1a, b). Authors concluded that

Fig. 15.1 Photographs of pouches after high pressure treatment. (**a**) picture of a PET/PP pouch after HP sterilisation treatment at 200 MPa (food simulant: tap water), *arrows* indicating regions of delamination; (**b**) detail of a delaminated region in PET/PP pouch with *arrows* highlighting the delamination zones; (**c**) picture of a PA/PP pouch after HP sterilisation treatment at 500 MPa (food simulant: tap water); (**d**) picture of a OPA/PP pouch after HP sterilisation treatment at 700 MPa (food simulant: solid carrots) (Fraldi et al., 2014)

the main cause of delamination could be attributed to the differences in the mechanical behaviour of the two films making up the multilayer structure and their dependence on temperature and pressure.

Pulsed electric field uses short pulses of electricity to inactivate microorganisms, causing minimal detrimental effects to the food quality and nutritional attributes. It can be used to preserve liquid food products that are normally pasteurised by thermal methods. New PEF treatments can be carried out after the packaging step, where conductive electrodes can be integrated into the package (Roodenburg et al., 2013). In this regard, Roodenburg et al. (2010) suggested that it was possible to get sufficient electric field inside a pouch made from any arbitrary packaging film. However, the direction of the applied electric field has a great influence on the electric field distribution and needs to be applied perpendicular to the surface of the film. Loss of electric field can occur due to variations in conductivity of food product and packaging material. Authors recommended choosing the film conductivity equal to the highest conductivity of the treated food product. The shelf life of foods packaged into plastics and processed by PEF treatment depends on the permeation of gas and water vapour through packages because a significant amount of food deterioration results from oxidation and changes in the water content (Akbarian et al., 2014).

15.5 Sustainable Fermented Food Packaging

15.5.1 Eco-design

One of the biggest challenges for the food packaging sector is to develop sustainable packaging systems that are able to minimise the environmental impact derived from packaging. In Europe, packaging represents the largest application sector for the plastics industry, with a 39.6 % of the total plastics demand. The amount of post-consumer plastics waste produced in Europe in 2012 was 25.2 million tonnes, from which 62 % were recovered (26 % recycled and 36 % recovered energy), and 38 % ended up in landfills (Plastics Europe, 2014). Although there is a positive trend observed in the recovery and recycling of plastics, other strategies are needed in order to promote sustainable packaging. Design is the most important and critical stage in the product development process with regard to producing better environmental outcomes, quality assurance, and consumer satisfaction (Park, Lee, & Han, 2014). Eco-design means the integration of environmental aspects into product design with the aim of improving the environmental performance of the product throughout its whole life cycle (European Union, 2009).

The key principles that need to be considered in the design or procurement of packaging to improve its sustainability are listed below (Australian Packaging Covenant, 2014).

- **Fit-for-purpose:** designed to meet market and consumer needs while minimising the net impact in a cost-effective way.

- **Resource efficiency:** designed to minimise the use of materials and other resources without compromising product quality and safety.
- **Low-impact materials:** designed to minimise the environmental and social impacts of materials and components.
- **Resource recovery:** designed to maximise its potential for recovery and recycling.

15.5.2 Successful Eco-design Strategies for Fermented Food Packaging

A selection of eco-design strategies addressed to minimise the environmental impact of packaging that have been successfully developed for fermented food products are listed below.

15.5.2.1 Source Reduction

Source reduction consists in reducing the amount of materials used to produce food packaging which results in a reduction of the amount of waste derived from packaging. Material reduction can be achieved by reducing packaging thickness, by using alternative materials or by reducing the number of packaging elements used (Australian Packaging Covenant, 2014). In this regard, Licciardello, Cipri, and Muratore (2014) proved that it was possible to reduce the thickness of the packaging used to pack industrial durum wheat bread (thermoformed bottom and lid) by about 20 % without affecting its shelf-life standards. In another study made in partnership with WRAP, Coca-Cola Enterprises and Beverage Can Makers Europe (BCME), it was proven that beer and cider aluminium cans can be lightweighted by 5 % without compromising the quality of the product (Waste & Resources Action Programme, 2008). Another strategy to reduce material consumption is to minimise the number of layers through the optimal combination of primary, secondary and transport packaging (Lewis, 2008). As an example, UK supermarket chain Sainsbury's replaced the packaging of its own-brand garlic bread consisting of a plastic sleeve (primary packaging) and a cardboard carton (secondary) with a polypropylene film pack. Product redesign resulted in a weight reduction of 70 % and improved logistics efficiency by 20 % (Holdway, Walker, & Hilton, 2002). The use of flexible materials as an alternative to rigid packaging materials such as metal cans and glass contributes to significantly reduce the weight impact, resulting in transport efficiency savings and overall improvement in environmental performance. For example, the use of a laminated pouch for beverage packaging results in a drop of the weight impact of the packaging during transport from 52 to 6 %. The weight impact relates directly to increased efficiencies in transportation and storage (Flexible Packaging Europe, 2012). Bonfire Winery has successfully launched its 1.5 L wine stand-up pouch consisting of a three-layer film produced by Curwood. This novel

design favours convenience thanks to a built-in tap, and represents an important material reduction compared to traditional glass bottles, Tetra Brik® or bag-in-box formats (Reynolds, 2014). Lightweighting can also be achieved through structural redesign of the package. In this sense, a number of UK-based, international lager beer brand owners took the challenge of making significant design changes to their bottles, achieving savings of 10,600 tonnes of glass. As an example, Cobra Beer successfully obtained a weight reduction of 20 % of the 660 mL bottle and Carlsberg UK a 17 % of the 275 mL bottle (Waste & Resources Action Programme, 2008).

15.5.2.2 Food Waste Reduction

Packaging design should be user-friendly in order to avoid food waste due to a difficult access. A recent study showed that in Sweden up to 10 % of the content of yogurt cartons (74 tons of yogurt) is wasted every year because consumers find it difficult to use all the yogurt contained in traditional packaging cartons. In order to solve this food wastage, the Swedish packaging company Ecolean has successfully developed and launched a flexible package particularly suitable for dairy products which enables the consumer to squeeze out nearly all of the content (Eliasson, 2008).

15.5.2.3 Use of Recycled Packaging Materials

The use of recycled materials as raw materials can significantly reduce the environmental impact of packaging. It has been estimated that the embodied energy saving per kg in the production of recycled glass, HDPE and PET is of 57 %, 79 % and 76 %, respectively, compared to virgin product (Lewis, 2008). In this sense, Siracusa, Ingrao, Giudice, Mbohwa, and Dalla Rosa (2014) estimated that the use of recycled PA resin instead of virgin PA in the production of bilayer (PA/LDPE) film bags for food packaging would lead to a reduction of about 15 % of the environmental damage.

15.5.2.4 Improvement of the Rate of Recycling by Changing the Materials

One way of promoting sustainable development is through recycling and the adoption of more environmentally friendly packaging. In the dairy sector, large yogurt producers have increased their rates of recycling and the type of packaging has changed. Some yogurt manufacturers such as Muller have changed the materials used for yogurt lids from aluminium to paper with a foil coating, which is more biodegradable and has reduced the amount of plastic used in each pot through material reductions achieved in pot walls and rim (Dewick, Foster, & Green, 2007).

15.5.3 Biodegradable Packaging Materials

In the search for environmental-friendly packaging polymers, a wide range of biodegradable materials are being exploited. Biodegradable polymers break down into natural compounds, therefore they would reduce the carbon footprint and make the system sustainable (Scott, 2002). Biodegradation takes place through physical decomposition and biological processes led by aerobic or anaerobic microorganisms, or under composting conditions. Biopolymers and biodegradable plastics are expected eco-friendly alternatives to petroleum-based polymers. Their implications in the preservation of the environment are, however, not uniform, and need to be carefully considered (Yates & Barlow, 2013). The choices in the group of eco-friendly polymers comprise biopolymers derived from natural sources which are biodegradable and compostable (Fig. 15.2), plastics made from renewable sources which are not necessarily biodegradable or compostable and synthetic plastics not based on renewable sources which are biodegradable (Siracusa, Rocculi, Romani, & Dalla Rosa, 2008). Coatings are applied in many food products to control moisture loss, to allow the selective exchange of gases or to control oxidative processes. In addition, they are linked to the possibility to achieve a personalised appearance and protection against microbial growth. In general, lipids are good in controlling water transmission, proteins provide excellent mechanical stability and polysaccharides are good oxygen barriers (Embuscado & Huber, 2009). Waxes have been used since the twelfth century to diminish water losses and to minimise mechanical damages in foods (Hardenburg, 1967). In particular, melted paraffin or paraffin-carnauba mixtures are used to cover cheese. The length of the fatty acid hydrocarbon chain is relevant to achieve acceptable barriers to water vapour (Morillon, Debeaufort, Blond, Capelle, & Voilley, 2002). Waxes can also decrease oxygen and carbon dioxide transmission and provide protection against microbial growth.

One of the most successful applications of biodegradable materials is meat casings. Casings must be strong but also shrinkable as they are important to determine

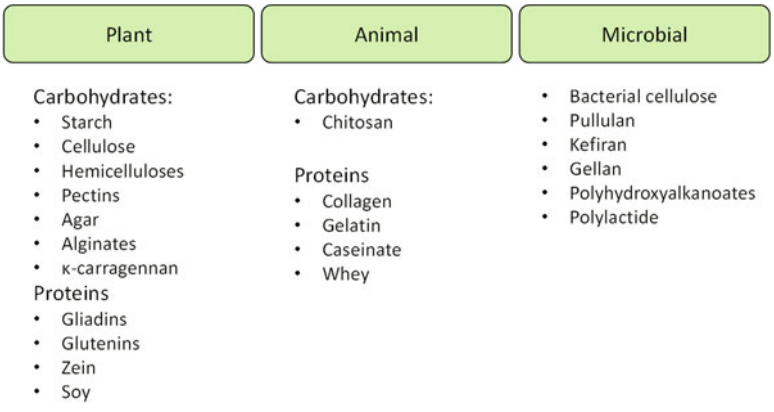

Fig. 15.2 Biopolymers with good perspectives in food packaging grouped by their origin

shape and size of the final product. Collagen casings are frequently used in dry and semi-dry fermented meat products because they present excellent stability and are permeable to smoke and moisture (Gomes, Santos, Almeida, Elias, & Roseiro, 2013). Collagen casings are edible in most applications. Another option for the production of uniform and strong casings is regenerated cellulose made from solubilised cotton or wood pulp (Nicholson, 1991). Cellulose has an ability to withstand curing and smoking processes which can substitute animal casings for fermented meat products. Regenerated cellulose edible casings allow the diffusion of oxygen, moisture, smoke and nutrients (Sreenath & Jeffries, 2011). Non-edible cellulose casings made of fibrous cellulose are also used commercially; however, fibrous cellulose is not permeable to smoke (Toldrà, 2014).

Carbohydrate and protein based packaging materials in fermented food products have also been investigated. For example, the potential of galactomannans as coatings of semi-hard cheeses was studied by Cerqueira et al. (2009, 2010). They reported that galactomannan-based coating can reduce gas transfer and cheese respiration rates, resulting in colour stability and extended shelf life. Kampf and Nussinovitch (2000) tested different films from k-carrageenan, alginate and gellan as coatings for semi-hard cheeses, finding a reduction in water vapour transmission rates and texture improvements. No, Meyers, Prinyawiwatkul, and Xu (2007) also showed a reduced microbial proliferation and staling in bread coated with chitosan. Blends of chitosan with starch (Mei, Yuan, Wu, & Li, 2013) and chitosan with sodium caseinate (Moreira, Pereda, Marcovich, & Roura, 2011) were effective in the storage of Mongolian cheese, cheese wraps and salami.

15.6 Active Packaging

The concept of active packaging refers to packaging systems where certain compounds have been intentionally added to the packaging material or in the packaging headspace to enhance the performances of the packaging itself, to improve food safety, quality and shelf life. Active packaging includes non-migratory strategies, the controlled migration of non-volatile agents and the emission of volatile compounds into the packaging headspace (Dainelli, Gontard, Spyropoulos, Zondervan-van den Beuken, & Tobback, 2008).

15.6.1 Oxygen Scavengers

Oxygen scavengers are able to reduce residual oxygen in food packaging environment to less than 0.01 % (Vermeiren, Heirlings, Devlieghere, & Debevere, 2003). The most extended commercial alternatives are sachets containing iron or ascorbic acid, and more recently, cerium and palladium. Many sorts of oxygen scavenger sachets are being commercialised under different trademarks. They have been recently reviewed

by Realini and Marcos (2014) and include Oxy-Guard™ (Clariant Ltd.), OxyCatch™ (Kyodo Printing Company, Ltd.), ATCO® (Standa Industrie) and FreshPax® (Multisorb Technologies, Inc.). Most of them find applications in meat, bread, bakery products and dry foods (Legrand, 2000). Salminen, Latva-Kala, et al. (1996) reported that the microbial shelf life of sliced rye bread was extended considerably by packaging with ATCO O_2 absorbers. However, individual sachets have limited applicability. They are not suitable for liquid foods and are not positively appreciated by consumers (Rooney, 1995). Extruded scavenging films, scavenging bottle closures or enzymatic O_2 scavengers would be preferred (Floros, Dock, & Han, 1997). Moisture-activated scavengers include a resin-bonded oxidable metal and oxidation promoters and fillers (Graff, 1998). In dry foods, UV-activated dyes can be incorporated (Nielsen, 1997). Glucose oxidase and catalase are frequently used in bottled beer or wines (Hardwick, 1995). Sulfate-based oxygen absorbers can also be incorporated into crown corks and plastic screw-on caps (Teumac, 1995), such as beer bottles. The incorporation of oxygen scavengers has opened new applications for low barrier materials such as PET. Chevron Phillips LLC and Sealed Air developed oxygen scavenger multilayer flexible films, in particular the OSP™ and Cryovac® OS2000 (Speer, Edwards, et al., 2009). OxyRx™ oxygen scavenging PET containers have been developed by Mullinix. Oxbar™ is a system developed by Carnaud-Metal Box (now Crown Cork and Seal) used especially in the manufacturing of rigid PET bottles for packaging of wine, beer, flavoured alcoholic beverages and malt-based drinks (Brody, Strupinsky, & Kline, 2001). Other materials such as EVOH, which suffers retort-shock and loses barrier, can also be reinforced with oxygen scavengers as in the case of EVAL™.

15.6.2 Ethanol Emitters

Modified atmosphere packaging of bakery products encounters a big problem associated to the large amount of pores in the matrix. They trap oxygen and favour the development of aerobic microorganisms (Galic, Curic, & Gabrić, 2009). To solve this problem, some companies offer ethanol vapour generators. In those systems, absorbed or encapsulated ethanol is released from sachets or laminate films when moisture is absorbed. The released ethanol is helpful to retard the growth of moulds in bread and bakery products, especially in products with high moisture, but also in semi-moist and dry products (Franke, Wijma, & Bouma, 2002). They have an additional antistaling effect. The addition of vanilla and other aromas can mask the off-ethanol flavour (Galic et al., 2009). Ethanol vapour generators may also be efficiently combined with oxygen absorbers. The combined systems have successfully extended the shelf life of bakery products such as sliced rye bread (Salminen et al., 1996), sliced wheat bread (Latou, Mexis, Badeka, & Kontominas, 2010) and durum wheat bread (Del Nobile, Martoriello, Cavella, Giudici, & Masi, 2003). Ethanol emitters such as Ethicap (Freund Industrial Co. Ltd), Oitech (Nippon Kayaku co, LTD), Ageless type SE (Mitsubishi Gas Chemical Co Ltd.) and some others reviewed by Day (2008) and Rooney (1995) are commercially available.

15.6.3 Carbon Dioxide Scavengers and Emitters

In modified atmosphere packaging, the headspace composition changes due to the higher permeability of polymers to CO_2 and the metabolic processes (Kanehashi, Kusakabe, Sato, & Nagai, 2010; Moller, Jensen, Olsen, Skibsted, & Bertelsen, 2000). In addition, CO_2 is highly soluble in fats and moisture; therefore, it might be required to replace it to avoid package collapse (Rao & Sachindra, 2002). CO_2 emitters in the form of sachets or labels usually contain ferrous carbonate or a mixture of ascorbic acid and sodium bicarbonate. Ascorbic acid absorbs oxygen and releases the equivalent amount of carbon dioxide (Waite, 2003). This technology has been applied to the storage of bread and bakery products, rice cakes and others. Mitsubishi Gas Chemical Co Ageless® is a carbon dioxide emitter. FreshPax R (Multisorb Technologies) has dual capabilities as oxygen scavenger and carbon dioxide emitter. On the other hand, carbon dioxide reacts with hydroxides to produce carbonates (Day & Potter, 2011), being the basis for the most commonly used carbon dioxide absorbers. Carbon dioxide scavengers are typically applied in the packaging of ground coffee because coffee produces considerable amounts of CO_2 that can cause the packaging to burst (Hurme, Sipiläinen-Malm, & Ahvenainen, 2002). The levels of carbon dioxide must also be controlled during storage of certain cheeses, such as Emmentaler cheese, to avoid unwanted blowing or the collapse of the package.

15.6.4 Moisture Absorbers

The accumulation of water in the package might reduce the shelf life of fermented food products affecting flavour, texture or accelerating the growth of moulds and bacteria. Several technologies have been developed based on the capabilities of desiccants such as silica gel, clay or lime. ATCO® (Standa Industrie) supplies a whole range of humidity absorbers. Multiform desiccants Inc. developed customised absorbers for moist, dry and refrigerated foods. FreshPax® S (Multisorb Technologies) are oxygen and moisture absorbers for bread, bakery, cheeses and other cultured dairy products that inhibit rancidity and retain the colour.

15.6.4.1 Antimicrobials and Antioxidants

Packaging polymers may play a supplementary role as carriers of antimicrobial or antioxidant molecules able to control pathogens and food spoilage microorganisms and to retard the oxidative processes (Bastarrachea, Dhawan, & Sablani, 2011). The action of antimicrobial additives and antioxidants may be controlled with tailored polymer blends, nanoclay incorporation, polymer crosslinking or chemical bonding (Duncan, 2011; Fernandez, Cava, Ocio, & Lagaron, 2008; LaCoste, Schaich, Zumbrunnen, & Yam, 2005). The impact of these technologies is however

limited due to the restrictive regulation concerning active packaging (European Commission, 2009). Besides, natural antimicrobials and antioxidants are sensitive to polymer processing temperatures and molecules required for chemical cross-linking are frequently toxic.

One of the most common surface preservatives in cheese and fermented meat products is natamycin (E235), a polyene macrolide antibiotic produced by *Streptomyces natalensis*. Natamycin is allowed to control mould development in cheese surfaces (El-Diasty, El-Kaseh, & Salem, 2008). In semi-hard and semi-soft cheeses, natamycin can be added to PVA coatings applied before ripening. Natamycin can also be added to collagen or cellulose casings of dry and fermented sausages to prevent mould growth in the casing, for example, under the trademark SANICO® (Laboratories STANDA). Many studies focus on the combination of natamycin with biopolymers. Gliadin films cross-linked with cinnamaldehyde and incorporated with natamycin were efficient to reduce moulds in cheese slices (Balaguer et al., 2014). In another study, a sol-gel processing of PLA with tetraethoxysilane and polyvinyl alcohol incorporating natamycin was tested on the surface of a semi-soft cheese with excellent results against mould spoilage (Lantano et al., 2014).

In edible films and coatings, preferred antimicrobials and antioxidants are bioactive natural compounds such as organic acids, essential oils, plant extracts, bacteriocins, enzymes or chitosan. Some examples illustrate the benefits of natural compounds in cheese edible coatings. Starch-based films coated with linalool, carvacrol or thymol were effective to eliminate *Staphylococcus aureus* inoculated on the surface of Cheddar cheese (Kuorwel, Cran, Sonneveld, Miltz, & Bigger, 2011). Cheese slices covered with edible pouches containing zein and oleic acid showed increased shelf life (Ryu et al., 2005). Ayana and Turhan (2009) used methylcellulose/chitosan films containing olive leaf extracts to control *S. aureus* growth in Kasar cheese. Sodium alginate coatings containing *Lactobacillus reuteri*, or lysozyme (E1105) and EDTA (E385) extended the shelf life of Fior di Latte cheese (Angiolillo, Conte, & Del Nobile, 2015; Conte, Gammariello, Di Giulio, Attanasio, & Del Nobile, 2009). Galactomannan and nisin (E234) showed positive results for Ricotta cheese preservation (Martins, Cerqueira, Souza, Carmo Avides, & Vicente 2010).

In addition, natural bioactive compounds in packaging materials can improve the quality of bread and bakery products. Chitosan coatings inhibited microbial growth and retarded bread oxidation and staling (No et al., 2007). Other authors reported that carvacrol and thymol incorporated in polypropylene were able to increase the shelf life of bread (Gutierrez, Escudero, Batlle, & Nerín, 2009). Similarly, cinnamaldehyde can be incorporated in gliadin films to increase the shelf life of sliced bread and cheese spreads (Balaguer et al., 2014). An active packaging with cinnamon essential oil combined with MAP was tested to increase the shelf life of gluten-free sliced bread. Active packaging was better than MAP alone, maintaining the sensory properties of gluten-free bread (Gutierrez, Batlle, Andújar, Sánchez, & Nerín, 2011).

The interest in metal-based micro- and nanocomposite materials is also growing. Among them, silver-based antimicrobials are widely used in the USA and Japan, and could grow in Europe after their inclusion in the provisional list of additives for use in food contact materials and in the list of surface biocides in the framework of

the Biocides Product (European Commission, 2011, 2012). Several masterbatches containing silver particles are being commercialised (Biomaster®, AgIon®, Irgaguard®, IonPure® and others). The applicability of silver as antimicrobial is however controversial since the concentrations necessary in foods are far above the recommended loads (Llorens, Lloret, Picouet, Trbojevich, & Fernandez, 2012). Many works report on applications in contact with fermented foods, mainly cheese. Agar, zein and PCL films reinforced with silver-montmorillonite have been tested against several microorganisms (Incoronato, Buonocore, Conte, Lavorgna, & Nobile, 2010). Among them, only agar loaded with silver nanoparticles was able to release silver ions due to the ability for water uptake, showing good perspectives to prolong the shelf life of Fior di Latte cheese (Incoronato, Conte, Buonocore, & Del Nobile, 2011).

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