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Sustainable Innovation in Food Product Design

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Sustainable Innovation in Food Product Design

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Preface

In the twenty-first century, manhood took, finally, the charge for environmental destruction and began striving to revert this situation to pass a healthy planet to future generations. Sustainability became one of the most important concepts of the actuality. A sustainable world will only be possible if people stops acting in a selfish way thinking only in the immediate profit of every action taken. The XII edition of CIBIA, Ibero-American Congress of Food Engineering, held for the first time in Faro, Portugal, at the University of Algarve, from 1 to 4 of July 2019, had as theme "Challenging Food Engineering as a Driver Towards Sustainable Food Processing." This theme was chosen since the impact of food losses on food security is a world striking concern. According to FAO (2015), in developing countries 40% losses occur at postharvest and processing levels, while in industrialized countries they occur at retail and consumer levels. Food engineering addresses this issue through research on sustainable alternative food processing technologies or engineering packaging for a sustainable food distribution chain. Moreover, sustainable innovation in food product design highly contributes as well to food sustainability.

This book is an output of CIBIA XII. It includes some of the best research works from oral or poster presentations selected by the editors. It is divided into 5 sections. The first section presents some of the latest developments in sustainable alternatives to chemical additives to extend meat and fish products' shelf life. The second section includes research work on the development of new sustainable food products. The third part relates to the development of plant-based products that can be an alternative to dairy foods and gluten-based cereals. The fourth section deals with consumer behavior related to new sustainable sources of nutrients from insects or seaweeds and the fifth part with the valorization of by-products from the food industry.

We wish that this book may inspire the readers to pursue this extremely challenging mission of turning the processing of food into a sustainable activity for the generations to come.

Faro, Portugal — Maria Margarida Cortez Vieira
Braga, Portugal — Lorenzo Pastrana
Santiago, Chile — José Aguilera

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The editors would like to express their gratitude and appreciation to the Springer publishing team for facilitating the steps needed to complete this challenging project. They would also like to thank all the chapters' contributors (authors) for their research work in food science and engineering to render the world more sustainable.

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Part I
Sustainable Alternatives to Chemical Additives to Extend Shelf Life

Chapter 1
Pitangueira Leaf Extracts as Alternative to Traditional Additives in Fresh Pork Sausage

Carla Giovana Luciano, Flávia C. Vargas, Larissa Tessaro, Marco A. Trindade, Lucas Arantes-Pereira, Andrezza M. Fernandes, and Paulo José do A. Sobral

1.1 Introduction

The use of natural additives to extend food shelf-life has been the subject of several research papers due to the new food conception adopted by consumers around the world, which aims at acquiring health and wellness from food products. Although alternatives to chemical additives is extremely desired by consumers, the use of plant preservatives is not a current reality yet. Besides that, natural antioxidants are less efficient and more expensive than synthetic ones (Fasseas et al. 2008).

Research about the effectiveness of natural additives is necessary due to the wide variety of bioactive compounds, and the lack of knowledge regarding extraction conditions, ways to maintain their stability, behavior and application in different food matrixes, quantities, economic viability, and sensory acceptance.

Meat is very susceptible to alterations during storage, which affect its color, odor, texture, and nutritional composition, mainly due to lipid and protein oxidation

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and spoilage (Pham et al. 2014). The utilization of plant-derived additives as alternatives to synthetic ones in meat products has been reported and reviewed recently (Falowo et al. 2014; Hygreeva et al. 2014; Karre et al. 2013; Pham et al. 2014; Qi et al. 2015). Extracts from less conventional plants, such as Pitangueira (Eugenia uniflora L) has attracted attention due to its bioactive properties (Vargas et al. 2016, 2019; Lorenzo et al. 2018).

In this chapter, the utilization of pitangueira leaf extracts as an alternative to traditional additives in fresh pork sausage is described. Two main experiments are reported: (1) several procedures to improve the amounts of bioactive compounds in extracts are described, and (2) fresh pork sausages with natural and/or traditional synthetic additives were assessed for their physical-chemical properties during 12 day-cold storage.

1.2 Quality Parameters of Pork Meat

1.2.1 pH, Oxidation and Microbiology

In the conversion of muscle into meat, pH falls from 7 to 5.5, approximately, in normal conditions (Greaser 2001; Trindade and Gressoni-Júnior 2008), reaching the isoelectric point of most meat proteins (approx. 5.5) and, therefore, lowering muscle water holding capacity (WHC) (Lawrie 2006). This water loss (around 1%) exudes myoglobin, causing meat to become lighter, softer and with moist aspect. These events promote desirable meat quality characteristics (color, texture, appearance, and yield). In this sense, final pH has a direct effect on water loss in meat and on quality parameters.

Lipid oxidation is the most significant reaction in all stored living tissues, negatively contributing to meat quality attributes (discoloration, off-flavor and others) (Pearson et al. 1983). Self-oxidation is the main lipid oxidation pathway (Berger and Hamilton 1995; Ramalho and Jorge 2006), which is influenced by light, pH, temperature, myoglobin concentration, presence of ions and unsaturated fatty acids (Chaijan 2008; Monahan 2000; Silva et al. 1999).

Meat color depends on the heme-globular myoglobin protein (Renerre 2000), that when exposed to oxygen for long periods oxidizes to metmyoglobin becoming brownish, which is associated with the lack of freshness by consumers (Gill 1996; Renerre 2000). Variations in myoglobin color are determined by the oxidative state and the type of molecule attached to the iron atom from the prosthetic heme group in this protein.

According to Faustman and Wang (2000), the interaction between lipid and myoglobin oxidation cannot be ignored since their products can act as pro-oxidants in muscle tissues (Baron and Andersen 2002; Chaijan 2008).

Meat quality can be altered by two groups of microorganisms: pathogenic, which can cause enteric or systemic diseases and fatal infections; and spoilage, which

alters the overall quality of meat (Marshall and Bal'a 2001; Nychas et al. 2008). The contamination of meat products by one of these groups can lead to major economic losses and/or serious risks to public health. Meat spoilage, together with protein and lipid oxidation are the main problems affecting the quality of meat products during shelf life.

1.3 Chemical Additives Used in Meat Products

Salt was the first food additive used to prevent meat spoilage and keep the product viable for later consumption (Pegg and Shahidi 2006). Another important additive in sausage formulation is nitrite (when allowed), which is involved in reactions that promote color, flavor and stability of cured meat products (Martin 2001; Pegg and Shahidi 2006). However, its use has been a cause of concern since the early twentieth century (Martin 2001), because of its toxicity in high concentrations, and its participation in the formation of carcinogenic compounds called nitrosamines (Pegg and Shahidi 2006). In 2015, this issue became intensified due to the classification of processed meat (e.g., sausages) containing nitrite, as 'carcinogenic to humans' by the International Agency for Research on Cancer (IARC), from the World Health Organization (WHO). The findings were on the bases of evidence of colorectal cancer and by positive association with stomach cancer (Bouvard et al. 2015).

Among the traditional food additives for meat products, the synthetic antioxidants butyl hydroxyanisole (BHA), butyl hydroxytoluene (BHT) and propyl gallate (PG) are commonly used (USDA 2015). Nevertheless, their use has also been questioned concerning safety, as they are suspect of having some toxic or carcinogenic effect (Soares 2002). Besides the mentioned synthetic antioxidants, other additives such as acidulants, acidity regulators, flavorings, colorants, color stabilizers, stabilizers, thickeners, flavor enhancers and humectants are also widely used in the preparation of meat products.

1.4 Plant Bioactive Compounds

Among plant bioactive compounds, the secondary metabolites are the most targeted by the food industry, as they usually contain phytochemicals of interest. Plant bioactive compounds can be divided into terpenes and terpenoids, alkaloids and phenolic compounds (Brielmann et al. 2006; Croteau et al. 2000), and the extraction of each type of compound is mainly dependent on its polarity. In this sense, not only the choice of solvent (Azmir et al. 2013), but also the determination of the extraction technique should be considered to obtain the desired bioactive compounds. Among the unconventional extraction techniques found in the literature, the ultrasound technique stands out for its speed, efficiency and low cost. This technique has been widely studied to obtain polyphenols and has shown positive results in increasing

carotenoid extraction from plant by-products (Wijngaard et al. 2012). In this type of extraction, the phenomenon called cavitation occurs occasioning disruption of plant cells, allowing better mass transfer and solvent penetration (Cavalheiro 2013; Sharmila et al. 2016).

1.4.1 Bioactive Compounds in Pitangueira Leaves

Due to the easy adaptation of pitangueira trees (*Eugenia uniflora* Linneus), this species is widely distributed in South American countries, and in several states in Brazil (Sobral et al. 2010). The Myrtaceae family comprises more than 5000 species, of which some has been characterized for antioxidant activity (Consolini and Sararubbio 2002; Nair et al. 1999; Sobeh et al. 2016).

The chemical profile of Myrtaceae, especially the species *Eugenia uniflora*, points to a range of phytochemicals such as flavonoids, quercetin, quercitrin, myricetin and myricitrin, as well as mono and sesquiterpenes (Amorim et al. 2009; Ogunwande et al. 2005; Schmeda-Hirschmann et al. 1987; Victoria et al. 2012).

Besides studies on pitangueira pulp and fruits, it seems that scientific interest on the properties of its leaves is increasing. According to Canabarro et al. (2019), pitangueira leaves are recognized as an important source of bioactive compounds of pharmaceutical and cosmetic interest. Some studies about the bioactive compounds from its leaves point to the possible existence of different chemotypes, which seems to be related to its fruit color biotypes (Mesquita et al. 2017; Costa et al. 2016). Mesquita et al. (2017) determined the profile of volatile compounds of fresh leaves from orange, red and purple fruit-biotype pitangueira trees, and suggested the existence of two varieties of pitangueira. On the other hand, Costa et al. (2016) identified three different types of compound profiles from pitangueira leaves without the existence of different varieties, but a high polymorphism instead.

Regarding phenolics, three main compounds have been identified in pitangueira leaf extract (Vargas et al. 2019), which are: myricitrin and quercetin 3-α-fucopiranoside as the major compounds in intermediate polar fraction (ethyl acetate) of the extracts; and quinic acid as the main compound in the polar fraction (Fig. 1.1).

1.4.2 Properties of Pitangueira Leaf Extracts

Because of its therapeutic properties, pitangueira leaves (Fig. 1.2) have been traditionally used in folk medicine in several tropical and subtropical countries to heal many health disorders and control biochemical blood parameters (Auricchio and Bacchi 2003; Ogunwande et al. 2005; Schumacher et al. 2015). This species is so popular that its use as a medicinal herb is provided by the Brazilian legislation (Anvisa 2005).

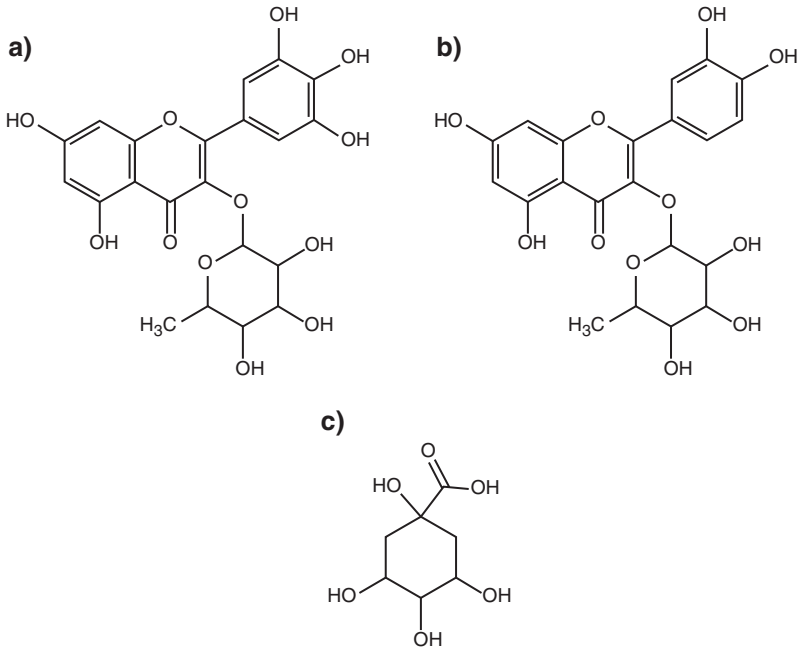

Fig. 1.1 Chemical formulae of phenolic compounds found by Vargas et al. (2019) in pitangueira leaf extracts: myricitrin (**a**), quercetin 3-α-fucopyranoside (**b**) and quinic acid (**c**) (Source: http://www.chemspider.com)

Fig. 1.2 Pitangueira leaves used to prepare the hydroethanolic extracts

The use of pitangueira in folk medicine inspired different studies on the properties of its leaves: it has been reported that pitangueira leaf extracts can inhibit the increase of plasma glucose and triglyceride (Matsumura et al. 2000) and its infusions presented anti-inflammatory effect in rats (Schapoval et al. 1994). Inhibitory

effect against enzyme xanthine oxidase has also been attributed to hydroethanolic extracts (7:3 EtOH: H_2O) of pitangueira leaves with no oral toxicity in mice (Schmeda-Hirschmann et al. 1987).

Also, aqueous extracts made of dried leaves from pitangueira have demonstrated higher in vitro antioxidant capacity when compared to rosemary extracts, considered a standard among the studied plants with antioxidant activity (Vargas et al. 2016). Besides that, *in vitro* antibacterial activity for negative and positive Gram bacteria has been reported by these authors. Moreover, extracts (60:40 EtOH: H_2O) from pitangueira leaves showed *in vitro* antioxidant and antimicrobial activity (Lorenzo et al. 2018).

Studies also reported that pitangueira leaf essential oil is a source of phenolic compounds with antioxidant (Garmus et al. 2014), antimicrobial and antifungal activity (Auricchio and Bacchi 2003; Ogunwande et al. 2005; Schapoval et al. 1994; Victoria et al. 2012).

To the best of our knowledge, very little information is found concerning the use of pitangueira leaf extracts to delay food spoilage and deterioration. It has been reported, though, that aqueous extracts were not able to improve lipid oxidation stability when applied in high concentration (1 mL/10 g) in refrigerated ground beef (Vargas et al. 2016). On the contrary, powder hydroethanolic extracts (60:40 EtOH: H_2O) at concentrations 250, 500 and 1000 mg/kg were as efficient as BHT to prevent lipid oxidation in pork burgers (Lorenzo et al. 2018). Besides meat products, pitangueira leaf extracts were tested in canola oil, and at 200 ppm it allowed canola oil stability by the inhibition of primary and secondary lipid oxidation processes (Vargas et al. 2019).

The use of pitangueira leaf extracts as an alternative to traditional additives to preserve fresh pork sausages during cold storage is reported below.

1.5 Pitangueira Leaf Extracts

1.5.1 Extraction Process of Pitangueira Leaves

The extraction process of pitangueira dried leaves followed several steps, in which different hydroethanolic proportions (water: ethanol—100:0, 20:80, 40:60, 60:40, 80:20, 0:100), ultrasound bath periods (15, 30 and 45 minutes), and temperatures (30, 60 and 80 °C) were assessed. Previous tests showed that the best plant material: solvent ratio for extract preparation was 1 g freeze-dried plant material to 10 ml solvent. These different extraction conditions allowed the understanding that higher ethanolic levels decreased all color parameters (L*, a* and b*) and occasioned higher Brix degree in extracts (Fig. 1.3).

The extraction process from pitangueira dried leaves that allowed the highest total phenolic was achieved with hydroethanolic proportion 40:60 (water: ethanol), 45 min in an ultrasound bath and magnetic stirring extraction at 80 °C. Table 1.1

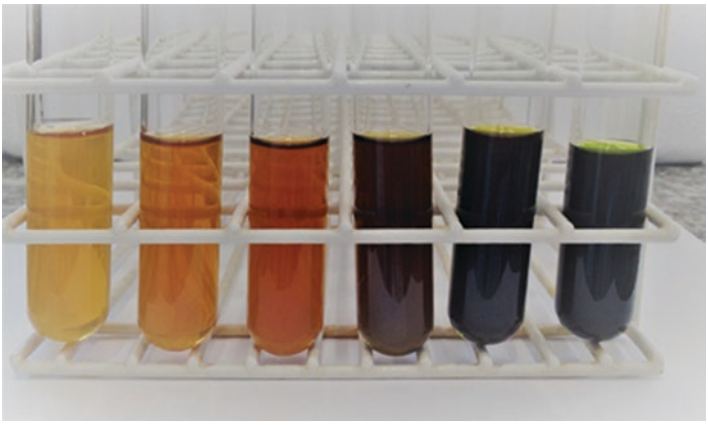

Fig. 1.3 Pitangueira leaf extracts prepared with different hydroethanolic proportions (water:ethanol—100:0, 20:80, 40:60, 60:40, 80:20, 0:100, from the left to the right), at 30 °C

Table 1.1 Extraction condition, total phenolic compounds as gallic acid equivalent (GAE), color parameters (CIE Lab), pH and total soluble solid content (°Brix) of the obtained pitangueira leaf extract*

Extraction conditions	Total phenolic Compounds**	L*	a*	b*	pH	°Brix
45' US 40:60 80 °C	0.29 ± 0.60	0.7 ± 0.4	1.5 ± 0.2	1.3 ± 0.7	4.87 ± 0.04	20.8 ± 0.3

*Means ± standard error. **g GAE/g dry matter

contains the main physical-chemical characteristics of the hydroethanolic extract chosen to be incorporated into fresh pork sausages.

1.5.2 Cytotoxicity of Pitangueira Leaf Extracts

Human Dermal Fibroblasts adult (HDFa) cells were exposed to different concentrations of PLE, and an effect on their viability after 48 h was found. From the concentration 1 mg/mL, a marked decrease in cell viability was noted. Through these results, it was possible to estimate the IC50, that is, the concentration of PLE that inhibited 50% of cell viability, which was 0.451 mg/mL.

Microscopic analysis (Fig. 1.4) of the plates showed a smaller number of cells after 24 h and 48 h culture at concentration 0.2 mg/mL, which corroborates the spectrophotometric analysis of cell viability performed by staining with yellow tetrazolium MTT (3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide). The presence of granules in the culture medium increased proportionally to the pitangueira extract concentration, starting from concentration 0.2 mg/mL.

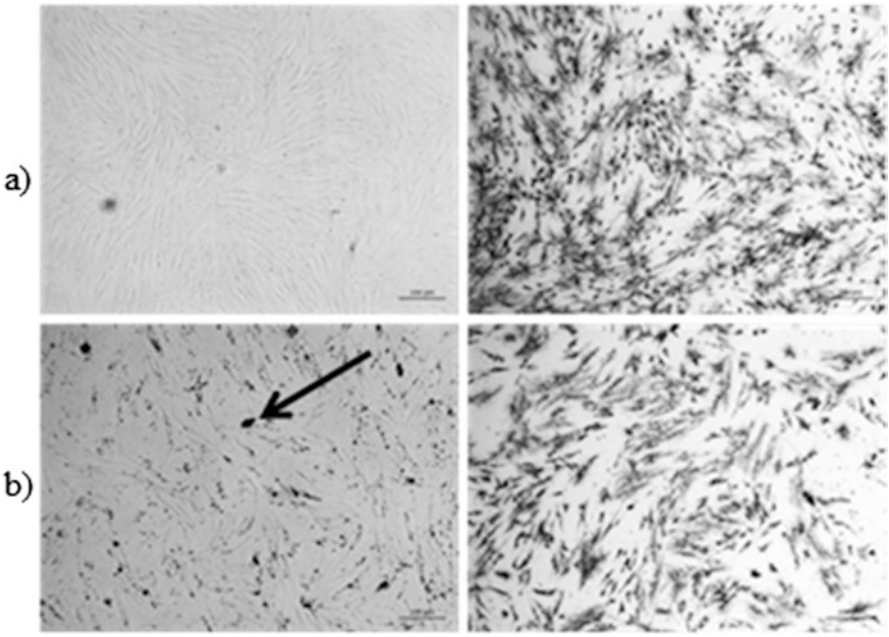

Fig. 1.4 Optical micrography of HDFa cells exposed or not to pitangueira leaf extracts after 24 h (left) and 48 h (right), stained with MTT (the arrow shows the presence of granules, resulting from the extract concentration): (**a**) control, (**b**) 0.2 mg/mL

Thus, the use of PLE is considered safe at moderate concentrations, since it did not present any alteration in cell viability at concentrations below 1 mg/mL. Likewise, the cytotoxic effect of pitangueira leaf ethanolic extract was studied by Cunha et al. (2016) in human leukocytes, where cell viability was determined microscopically in cell suspensions containing extract concentrations from 0.001 to 0.48 mg/mL. Nevertheless, these authors did not observe an alteration in cell viability in human leukocytes exposed to pitangueira leaf ethanolic extract. Similarly, pitangueira leaf methanolic extracts used by Braga et al. (2007) did not show cytotoxicity to J774 murine macrophage cells at a concentration of 0.25 mg/mL. This difference was possibly due to differences in the type of cell, type of extraction and experimental conditions.

1.5.3 Antioxidant Activity of Pitangueira Leaf Extracts

PLE showed high antioxidant activity by radical scavenging methods, namely $DPPH^{\bullet}$ (2,2-diphenyl-1-picryl-hydrazyl) assay, described by Brand-Williams et al. (1995), and the $ABTS^{\bullet+}$ (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) assay, described by Re et al. (1999). These extracts were able to inhibit DPPH

radical absorbance significantly, demonstrating a high percentage of scavenging ability (81.5 ± 2.6%) when used at concentration 0.4 mg/mL.

As a matter of comparison, Schumacher et al. (2015) also evaluated the antioxidant activity by assessing the percentage of radical sequestration of pitangueira leaf extracts and reported radical inhibition of 39.9 ± 1.9, 27.6 ± 2, and 34.2 ± 1.5% at concentration 40 mg/mL for aqueous, methanol/acetone and ethanol extracts, respectively. Besides that, Lorenzo et al. (2018) also reported high antioxidant activity of pitangueira leaf extracts by the DPPH radical scavenging assay expressed as EC50 (0.242 ± 0.014 mg/mL). Likewise, Victoria et al. (2012) reported the antioxidant activity of pitangueira leaf essential oil as EC50 of 0.83 mg/mL.

Concerning the ability of PLE in scavenging the $ABTS^{\bullet+}$ radical expressed as TEAC (mg Trolox equivalent/g DM extract), the antioxidant activity achieved in the current study was 93.73 ± 7.9 mg Trolox/g DM. Other authors also reported the antioxidant activity of pitangueira leaf extracts using the $ABTS^{\bullet+}$ radical assay: Lorenzo et al. (2018) also assessed pitangueira leaf extracts and obtained 570.97 mg Trolox/g DM. In turn, Schumacher et al. (2015) found a statistical difference between the aqueous (8,9 mg Trolox/g sample) and ethanol (6,2 mg Trolox/g sample) extracts ($p < 0.01$).

The results on the determination of the antioxidant activity obtained in this study allowed the understanding that the use of ultrasound-assisted extraction, followed by magnetic stirring using higher temperatures (80 instead of 30 or 60 °C), occasioned better phenolic compound extraction and thus, increased antioxidant power of PLE.

For all the revised studies about pitangueira leaves, it is considered common sense that little is known about the bioactive compounds responsible for the antioxidant activity of the species. In general, this property is attributed to the synergic effect of major and minor compounds present in extracts of the species' leaves. Nevertheless, some of the substances present in this kind of extract may not have the desired bioactivity able to prevent food oxidation, and in this sense, the hydroethanolic extracts were fractionated in an attempt to identify which fractions have better antioxidant activity.

Assays to assess the antioxidant power of fractions (nonpolar, intermediate and polar) of PLE were carried by the capture of the radical $ABTS^{\bullet+}$ and as a result, only the polar and intermediate fractions (obtained by the use of ethyl acetate) showed significant antioxidant power (Table 1.2).

Table 1.2 Antioxidant activity of fractions from pitangueira leaf extracts by the $ABTS^{\bullet+}$ radical scavenging assay

Fractions	TEAC* (mg Trolox/mg MS extract)
Nonpolar	0.4^{b}
Intermediate	79.4^{a}
Polar	112.3^{a}

*TEAC—Trolox equivalent. $^{a–b}$Means followed by equal letters in the same row do not differ statistically by Tukey test at 5%

As a follow-up to the antioxidant assay from the fractions of the pitangueira extracts, the identification of the main compound from polar and intermediate fractions was carried out by HPLC-DAD-MS, as reported in Vargas et al. (2019), whose results revealed that main compounds found in the intermediate fraction were quinic acid in polar fraction and myricitrin and quercetin 3-α-fucopiranoside in intermediate fraction.

1.5.4 Antibacterial Activity of Pitangueira Leaf Extracts

PLE was also submitted to antibacterial activity analysis and presented very satisfactory results for the sensitivity tests (inhibition zones—IZs), minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). Results from these tests were reported by Lorenzo et al. (2018), and according to them, all strains were equally inhibited (IZ) by the extract ($p > 0.05$). Except for *E. coli*, the growth of all bacterial strains (*Bacillus cereus* ATCC 14579; *Escherichia coli* ATCC 25922; *Pseudomonas aeruginosa* ATCC 15442; Salmonella spp. ATCC 13076, and *Staphylococcus aureus* ATCC 25923) was inhibited (MIC) by the use of different concentrations of PLE. Likewise, Souza et al. (2004) reported an inhibitory effect of pitangueira leaf methanolic extract for *Staphylococcus aureus* and *Bacillus subitilis* strains, and whereas no antibacterial action was found for *E. coli*.

1.6 Shelf Life of Fresh Pork Sausages with Pitangueira Leaf Extracts

Our experiment regarding the use of pitangueira leaf extracts as an alternative to traditional additives in fresh pork sausages is described as follows. In this study 4 sausage formulations have been prepared: Positive Control, with sodium nitrite and sodium erythorbate; Erythorbate Control, with sodium erythorbate and reduced-sodium nitrite; Negative Control, with reduced-sodium nitrite; and Extract, with reduced sodium nitrite and pitangueira leaf extract in levels defined by antibacterial assays. To assess the use of pitangueira leaf extract as an alternative additive, the shelf life of fresh pork sausages was conducted for 12 days in cold (4 ± 1 °C) storage in which instrumental color, pH, water activity, lipid oxidation, microbiology, and sensory acceptance were monitored throughout time.

1.6.1 *Instrumental Evaluation of Color, pH, and Water Activity of Sausages with Pitangueira Leaf Extract*

Instrumental color parameters L*, a* and b* of the studied sausages were analyzed to verify the effect of the treatments and the storage time, or even the interaction between these factors, in the referred parameters.

For parameter L* (luminosity), no significant interaction was observed between the factors under study, but there was a significant effect of the treatment factors and storage time in isolation. During the cooling period, the sausage luminosity, regardless of the treatment, decreased during the first 8 days, from 63.15 to 58.36, increasing slightly on the 12th day of storage, reaching 61.74. This variation in the luminosity of sausage samples may be due to the different physicochemical reactions that occur during storage. Similar results were obtained by Baldin et al. (2016) in fresh pork sausages added, or not, with microencapsulated jabuticaba peel extract. These authors did not observe an interaction between the factors and treatment time, but there were changes over time, reported to decrease L* parameter for all treatments ($p < 0.05$). Among the tested treatments, the lowest average luminosity was obtained for the samples of the extract treatment (58.04 ± 2.2), indicating the change of this parameter occasioned by the color of the plant extract.

In parameter a*, which measures the intensity of the colors green to red, a significant interaction was observed between treatment factors and storage time. In other words, the factors acted together in determining the intensity of a* in the sausages under study. From what can be seen in Fig. 1.5, the red intensity of fresh sausage samples from all treatments showed, in general, a growing trend over the storage

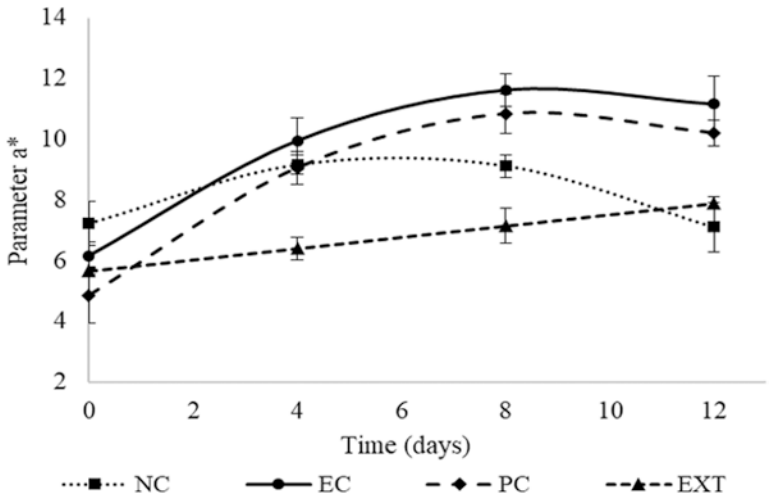

Fig. 1.5 Evolution of color parameter a* from fresh pork sausage samples from different treatments during refrigerated (4 °C) storage (*PC* positive control, *EC* erythorbate control, *NC* negative control, *EXT* extract)

period, which was expected by the presence of nitrite in all formulations. The inclusion of pitangueira leaf extracts in fresh sausages caused lower values of parameter a*, which is probably due to the green coloration of the plant extract, as mentioned above.

The other treatments presented similar behavior, with a slight decrease in this shade after the 8th day of storage, most probably due to the beginning of the myoglobin oxidation process. However, there was a more pronounced decrease in the red intensity of the negative control treatment samples, also from the 8th day of the sampling period. This behavior was already expected since the negative control treatment did not contain antioxidant substances, containing only a reduced-sodium nitrite content. Thus, protein oxidation was more accelerated in this treatment.

Regarding parameter b*, which measures the intensity of the colors blue to yellow, no effect of time or treatment was observed, nor the interaction of these factors, indicating that the intensity of yellow of fresh sausages was unchanged during the experiment. Although statistical analysis showed treatment effect and time for sausage samples regarding water activity, little change was generally observed, so that it remained around 0.97 for samples from all treatments throughout the entire period. These values are as expected, as it is a fresh meat product. Concerning pH, a significant interaction between treatment and factors storage time was observed, and in general, there was a tendency for this variable to increase (Fig. 1.6).

It was observed that the samples of the erythorbate control and negative control treatments showed both values and variations very similar over the period, which was expected since neither of them has any additives with antibacterial action. The evolution of the mean pH values of the extract treatment samples was also very

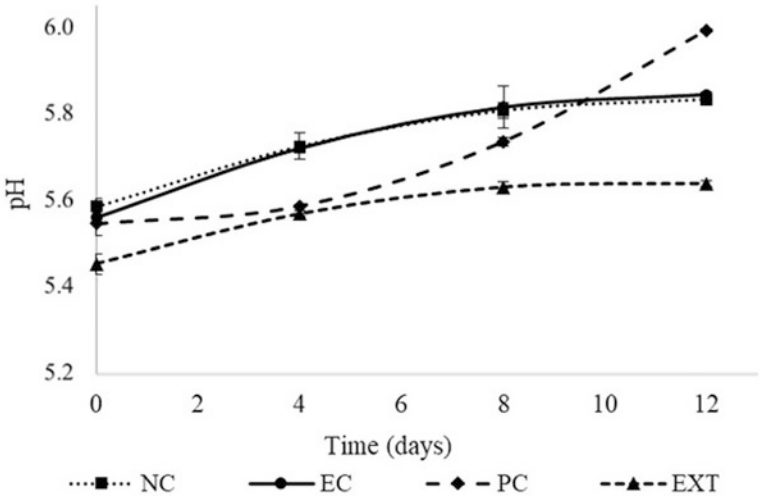

Fig. 1.6 Evolution of pH of fresh pork sausage samples from different treatments during the refrigerated (4 °C) storage (*PC* positive control, *EC* erythorbate control, *NC* negative control, *EXT* extract)

similar to the latter, but with lower values, probably caused by the acidity of the plant extract itself, whose pH was 4.87. In turn, the positive control sausages kept their pH below that of the negative and erythorbate control samples for most of the shelf time, as expected. Lower pH values were also observed by Lorenzo et al. (2014), in ground pork with natural extracts of green tea and grape seed, a fact that the authors attributed to the properties of bioactive compounds formed by acid groups present in the extracts. Thus, it is also considered that the presence of bioactive compounds such as chemical acid, identified in the phenolic compounds of plant extracts (Vargas et al. 2019), may have influenced the low pH values of the extract treatment sausages.

Generally, the pH of the treatment samples all showed an increase over the storage period, which according to Nychas et al. (1998) can be due to the action of many species of bacteria that produce ammonia by metabolizing amino acids, promoting the pH increase of deteriorating meat products.

The lower pH values of the sausages in the extract treatment presents a great advantage both in the control of microbiological growth, as in some meat product quality attributes as greater water retention capacity with a consequent increase in juiciness and yield, a decrease of cooking and freezing weight losses and improved texture.

1.6.2 Lipid Oxidation of Fresh Pork Sausages with Pitangueira Leaf Extract

Lipid oxidation, measured by TBARS method, in fresh pork sausage samples, showed significant interaction ($p < 0.05$) between factors, which means that responses to this variable were dependent both on time and treatment (Fig. 1.7). As can be seen, except for extract treatment, there was a growing trend for TBARS values in fresh sausage samples with an overall average ranging from 0.65 to 1.2 mg MDA/kg sample, namely, lipid oxidation of the mentioned treatments increased during shelf life.

In the raw material used to prepare the sausage formulations, some degree of lipid oxidation was detected by the presence of secondary compounds from the oxidative lipid process (malondialdehyde-MDA) in relatively high quantities on day 0. However, the increase of such compounds and thus MDA values throughout storage time was expected since chemical reactions as oxidation still occur in meat samples under refrigeration.

Among the tested sausage formulations, extract treatment was the only one capable of maintaining lipid oxidation values at initial levels (Table 1.3), showing minimal propagation of this chemical reaction, which is promoting better protection of the lipids present in the fresh pork sausages with pitangueira leaf extract. This is a very important finding because it demonstrates the high antioxidant capacity of pitangueira leaf extracts when used in this type of meat product stored under

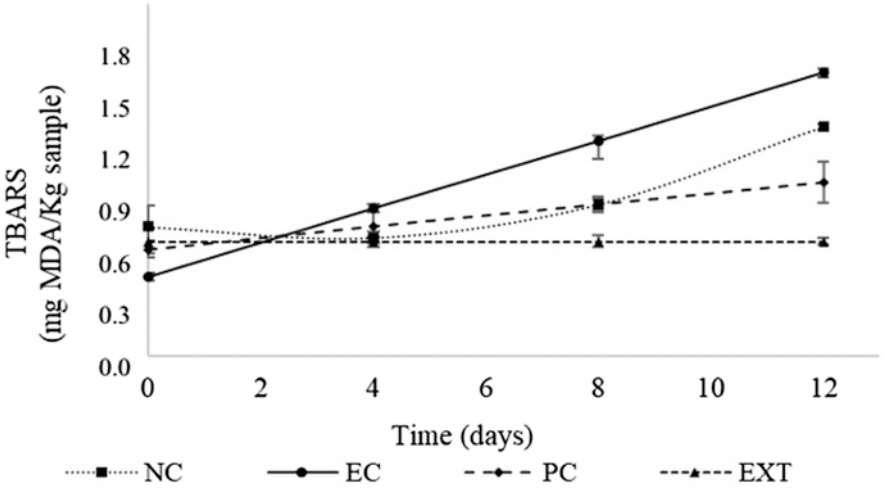

Fig. 1.7 Evolution of lipid oxidation by TBARS in fresh pork sausage samples from different treatments during the refrigerated (4 °C) storage (*PC* positive control, *EC* erythorbate control, *NC* negative control, *EXT* extract)

Table 1.3 Average values of lipid oxidation (TBARS) of fresh pork sausages from different treatments under cold storage of 12 days

Sausage treatment	TBARS (mg MDA/kg sample)*
Negative control	0.94 ± 0.31^{ab}
Erythorbate control	1.10 ± 0.60^{a}
Positive control	0.84 ± 0.19^{bc}
Extract	0.68 ± 0.13^{c}

*Means ± standard error. [a–c]Means followed by equal letters in the same row do not differ statistically by Tukey test at 5%

refrigeration. Besides that, the fact that the extracts protected sausages from lipid oxidation corroborates with results from the antioxidant activity analysis performed in these extracts (see Sect. 1.3).

Also, it should be noted that since samples were already oxidized at the beginning of the storage period, the antioxidant activity of extracts showed an ability to act mainly during the propagation phase of lipid oxidation (Ramalho and Jorge 2006). This capacity PLE was probably due to the presence of phenolic acids, such as quinic acid, which have not only the ability to chelate metals that participate in the generation of reactive species and hence initiate lipid oxidation, but also the ability to control the propagation phase of the oxidative process by donating electrons to reactive species already found in the environment (Shahidi et al. 1992; Soares 2002). These results demonstrate the effectiveness of PLE as natural antioxidants in chilled pork products.

The evolution of TBARS in sausages from treatment positive control was similar to treatment extract, showing a slight increase over time, as expected. Treatments

erythorbate and negative control had prominent growth at the end of the storage period, demonstrating little protection from lipid oxidation of fresh pork sausages during the 12-day cold storage.

1.6.3 Microbiology of Fresh Pork Sausages

In these assays, microbiological plates from day 12 were dismissed since the amount of colony-forming units (CFU) was too high, hindering counts for all the tested microorganisms. Nevertheless, little variation between treatments was observed for *Pseudomonas* spp., where the increasing tendency of colony amount has been observed for this bacteria species (Fig. 1.8). To some extent the increase in *Pseudomonas* spp. counts were expected, as this is one of the major spoilage bacteria in refrigerated meat (Marshall and Bal'a 2001). Therefore, no antibacterial effect was observed for *Pseudomonas* spp. growth with the use of additives from the different treatments in the current experiment.

Counts of psychrotrophic also showed an increasing trend for all treatments (Fig. 1.9). Nevertheless, sausages from treatment positive control had lower counts throughout the storage period, as expected for a synthetic additive. Although sausage samples already had high initial counts (day 0), the antibacterial action of sodium nitrite additive in sausages from positive control seemed sufficient to contain the increase of psychrotrophic during the storage period, thus evidencing its efficacy in refrigerated meat products. On the other hand, treatments erythorbate and extract were not efficient to control this species' growth.

Total coliform counts in sausage samples (Fig. 1.10), presented, in general, a decreasing tendency during cold storage. This type of behavior can be expected in food products during cold storage due to nutrient competition caused by the increase

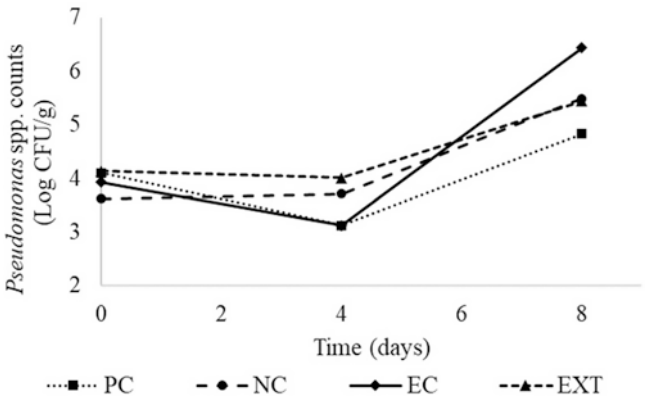

Fig. 1.8 Evolution of *Pseudomonas* spp. counts in fresh pork sausage samples from different treatments during the refrigerated (4 °C) storage (*PC* positive control, *EC* erythorbate control, *NC* negative control, *EXT* extract)

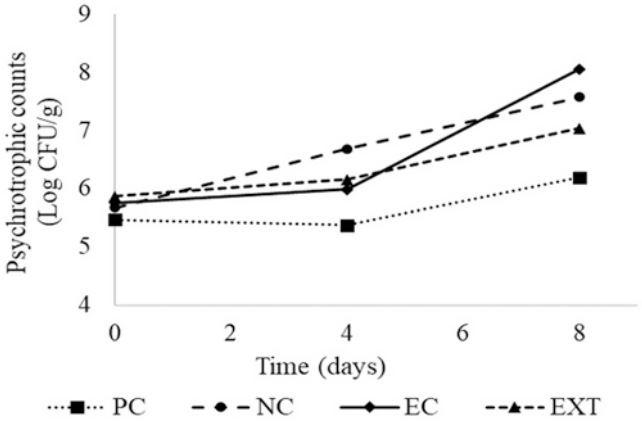

Fig. 1.9 Evolution of psychrotrophic counts in fresh pork sausage samples from different treatments during the refrigerated (4 °C) storage (*PC* positive control, *EC* erythorbate control, *NC* negative control, *EXT* extract)

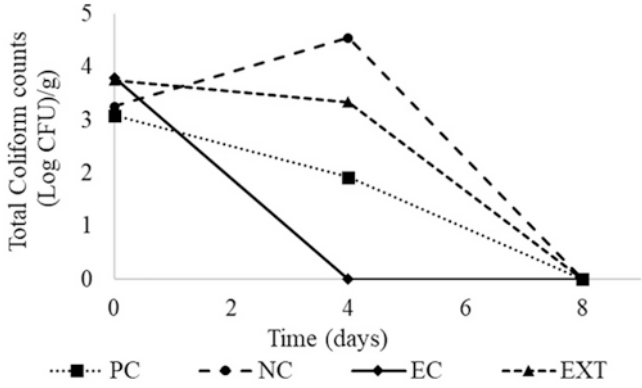

Fig. 1.10 Evolution of total coliform counts in fresh pork sausage samples from different treatments during the refrigerated (4 °C) storage (*PC* positive control, *EC* erythorbate control, *NC* negative control, *EXT* extract)

of spoilage microorganisms, such as *Pseudomonas* and psychrotrophic, which has been confirmed by the evolution of such species (Fig. 1.8 and 1.9).

A variation in total coliform counts between samples from the different treatments during the storage period was observed, especially on day 4, when CFU from negative control samples increased, probably because of the lack of substances capable of inhibiting this species' growth. By that time, the competition for nutrients was probably not very high, considering the population of *Pseudomonas* and psychrotrophic bacteria.

Although it is known that the initial microbiological load is related to the physiological state of the animal and sanitary conditions during slaughter (Marshall and

Bal'a 2001; Nychas et al. 2008), the contamination during processing of meat products directly influences its microbiological status, especially considering total coliform population. In the present experiment, the use of additives (traditional or natural) has influenced the counts of the total coliform population from treatments positive control, erythorbate control, and extract.

1.6.4 Sensory Acceptance of Pork Sausages with Pitangueira Leaf Extracts

Sensory acceptance of cooked pork sausages was assessed to verify consumers' preference, duly approved by the Ethics Committee of Faculdade de Zootecnia e Engenharia de Alimentos from Universidade de São Paulo (FZEA/USP CAAE 74595917.8.0000.5422, approval 2.247.424). Consumers (n = 106) used a 9-points hedonic scale (1—dislike extremely and 9—like extremely) to evaluate attributes taste, aroma, color, texture and global acceptance from sausages.

For all the tested attributes significant differences were found (Table 1.4), in which sausages from treatment extract received lower grades, in general. Grades for attribute taste were around 7 (like moderately) for all the tested treatments, which means that consumers approved this attribute. Sausages from treatments positive control received better grades than treatment extract, while sausages from erythorbate and negative control had intermediate values.

For the aroma attribute, sausages from negative control and extract had similar grades, while samples from positive control and erythorbate control received higher grades than treatment extract. This indicates that the presence of extracts into sausages affected sausages' aroma negatively. In some cases, a smell of green grass was reported by consumers, which may have influenced the lower means from this treatment.

Sausages from treatment extract received the lowest mean grades for color attribute. Consumers complained that color was too dark, which has also been detected by the laboratory team during the process and storage of raw sausages from this treatment. This difference in sausages' color is due to extracts' dark green appearance (Fig. 1.3) and can be understood as an issue that needs to be solved when using

Table 1.4 Sensory acceptance notes of fresh sausages*

Treatment**	Taste	Aroma	Color	Texture	Global acceptance
PC	7.4 ± 1.2^{a}	7.0 ± 1.4^{a}	7.2 ± 1.4^{a}	6.8 ± 1.3^{ab}	7.1 ± 1.2^{a}
NC	7.1 ± 1.4^{ab}	6.7 ± 1.4^{ab}	6.5 ± 1.5^{b}	6.7 ± 1.4^{b}	6.7 ± 1.4^{ab}
EC	7.2 ± 1.3^{ab}	6.9 ± 1.3^{a}	6.8 ± 1.7^{ab}	7.1 ± 1.5^{a}	7.0 ± 1.3^{a}
EXT	7.0 ± 1.4^{b}	6.5 ± 1.5^{b}	5.5 ± 1.9^{c}	6.6 ± 1.6^{b}	6.5 ± 1.4^{b}

*Means ± standard error. **_PC_ positive control, _NC_ negative control, _EC_ erythorbate control, _EXT_ extract. [a-c]Means followed by equal letters in the same row do not differ statistically by Tukey test at 5%

this kind of natural additive. On the other hand, considering the new trend in food consumption, in which natural-based products are well accepted by consumers even when traditional appearance is altered, it is believed that by justifying the use of plant extracts in sausage formulations this matter will no longer exist.

The texture of pork sausages seemed not to be affected by the use of extracts since no difference was found among samples from negative control, positive control and extracts. About global acceptance, sausages from treatment extract were less accepted than the ones from treatments positive and erythorbate control, which has been attributed mainly to the influence of aroma and color in sausages containing pitangueira leaf extracts, as samples were darker and with a grass-like smell.

The findings from the current experiment were very important to better understand how consumers react when a plant-based additive is applied to a meat product, and thus support meat industry decisions towards the shift from chemical to natural additives in meat products.

1.7 Final Remarks

Among the tested procedures to obtain phenolic compounds from PLE, the extraction method developed in this study, using 1 g freeze-dried plant material/10 mL solvent (40:60, EtOH: H_2O), assisted by ultrasonic treatment for 45 minutes, followed by magnetic stirring at 80 °C, provided the best results not only for the in vitro antioxidant capacity of these extracts, but also to its antibacterial activity.

The presence of PLE in formulations of fresh pork sausages decreased product's luminosity and redness, nevertheless, it promoted excellent lipid protection, evidencing the remarkable antioxidant capacity of this extract. PLE did not influence the microbial growth of fresh pork sausage in the tested proportions. Less global acceptance of sausages containing PLE seems to be related to the dark-green color and grass-like odor from the extract.

Considering that the yellowish polar and intermediate fractions of PLE showed antioxidant activity and that the dark green non-polar fraction showed almost none antioxidant activity, the use of only active fractions (polar and intermediate) in meat products to promote better brightness, red intensity and consumer acceptance is suggested. Another suggestion is to use an activated charcoal layer to remove pigments from PLE before its use in meat products.

The utilization of PLE in fresh pork sausage has been described thoroughly in this chapter, and according to results obtained and the consulted literature, this extract showed a huge potential to be used as a suitable natural alternative to chemical additives in meat products under cold storage.

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Chapter 2
Inhibition of Microbial Growth in Bovine Meats Surface by Combined Physical Agents and Natural Additives

Mariana F. Blanco, Ana J. Amasino, Irene Pena, Gladys Laporte, Pablo Sota, Daniela Olivera, and Fernanda C. Cárdenas

2.1 Introduction

Meat is one of the main foods consumed worldwide. Its quality has always been important to the consumer, and it is an especially critical issue for the meat industry in the twenty-first century. As consumer demand for high-quality meat is increasing in most countries, the meat industry should consistently produce and supply quality meat that is tasty, safe and healthy for the consumer to ensure continued consumption of meat products (Joo et al. 2013). Due to its natural composition, beef is a product that can be contaminated with spoilage microorganisms and pathogens because of its processing and handling (Fernández Blanco et al. 2019). At the same time, by the inherent characteristics of each species, often these microorganisms survive and sometimes grow at refrigeration temperatures during processing and storage of this food (Olivera and Coll Cárdenas 2012). Therefore, the control of microorganisms in meats is a critical point. The inhibition of spoilage microorganisms is mainly the process used in the preservation of meat, although other preservation methods are sought to minimize other deterioration changes such as color and oxidative changes (Zhou et al. 2010).

Despite modern improvements in slaughter hygiene and food production techniques, food safety is an increasingly important public health issue. There is

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therefore still a need for new methods of reducing or eliminating food-borne pathogens and the development of spoilage microorganisms, when possible in combination with existing methods.

Wherewith, to extend the period of refrigerated storage, which is the most common method used for preserving fresh meat and meat products, the application of the concept of barriers it is an interesting alternative to study. In this sense, by combining different methods: irradiation with ultraviolet light, cooling temperatures, pH decrease, and the addition of natural additives such as essential oils, are some of the nonthermal technologies that can be considered to extend the microbiological shelf life, which when operated together will have a greater activity that by doing it separately.

The objectives of this chapter are, first, to review some applications of these technologies in fresh beef to extend their shelf life and then present the results of the effect of the combined application of physical agents and natural additives on the growth kinetics of spoilage microorganisms in bovine meats surface.

2.1.1 UV-C Light

Although refrigeration has long been the traditional method of preserving fresh beef, this technology offers a short shelf life, ranging from 3 to 5 days when beef is stored at 4 °C (Fernández Blanco et al. 2016). The food industry needs technological alternatives that can be used as tools to increase the shelf life of meat.

Ultraviolet light (UV light) is one such non-thermal technology. Ranges in wavelength from 100 to 400 nm and is divided into UV-A (315–400 nm), UV-B (280–315 nm), UV-C (200–280 nm) and UV-V (100–200 nm, vacuum UV range) (Guerrero Beltran and Barbosa Canovas 2004).

Particularly, UV-C in the range of 250–260 nm is lethal to bacteria, viruses, protozoa, mycelial fungi, yeasts, and algae. It is a powerful bactericidal agent; not ionizable and, when absorbed by proteins and nucleic acids, affects the genetic material of microorganisms, inducing changes in cell multiplication and viability (Haughton et al. 2011). Most microorganisms absorb UV-C radiation at 254 nm, which causes electron displacement and DNA breakdown, preventing the multiplication of vegetative pathogenic microorganisms (Engo et al. 2015). The susceptibility of different microorganisms varies according to the characteristics of their cell wall, proteins, and DNA, which is why specific doses will be required to inactivate them (Guerrero Beltran and Barbosa Canovas 2004). In addition, the sensitivity of food to UV light is a function of intrinsic factors such as the chemical composition and external factors such as the UV light intensity, the available oxygen, the temperature and the presence of packaging barriers.

Also, UV-C between 220 and 300 nm is approved by the FDA as a means of controlling food spoilage microorganisms (US Food and Drug Administration 2007).

Several kinds of meat may be UV-C treated on the surface for reduction of the microbial load before refrigeration. For beef, its effectiveness has been

Table 2.1 Summary of studies of the effect of UV-C light on reduction of microorganisms in fresh beef

Sample	Target microorganism	UV dose	Log reduction	References
Beef steaks	*Pseudomonas, Micrococcus, and Staphylococcus*	0.15 W·s·cm^{-2}	2 log cycles	Stermer et al. (1987)
		0.50 W·s·cm^{-2}	3 log cycles	
Beef slices	Mesophilic, psychrotrophic	5.40 W·s·cm^{-2}	2–3 log cycles	Kim et al. (2014)
	Coliform and gram-negative		1 log cycles	
	L. monocytogenes, S. typhimurium, and *E. coli O157*: H7		Decreased to 84.64, 80.76, and 84.12%, respectively	
Beef slices	Total bacterial count (TBA)	12.7 W·s·cm^{-2}	1 log cycles	Hassan et al. (2015)
		25.5 W·s·cm^{-2}	2 log cycles	
		38.2 W·s·cm^{-2}	3 log cycles	
Ground beef	*Salmonella*	2.4 × 10^{-2} W·s·cm^{-2}	1 log cycles	Yeh et al. (2018)
		2.4 × 10^{-2} W·s·cm^{-2} and bacteriophages	2 log cycles	
Beef slices	Shiga toxin-producing *E. coli (STEC)*	0.59 W·s·cm^{-2} and ozone.	1.49 log cycles	Kalchayanand et al. (2020)
	Salmonella		1.33 log cycles	
	Listeria monocytogenes		1.14 log cycles	
	Total aerobic bacteria		1.23 log cycles	

demonstrated by some authors (Table 2.1). Stermer et al. (1987) informed that UV-C light (253.7 nm) was effective in reducing the number of surface bacteria in fresh meat. A radiation dose of 0.15 W s cm^{-2} reduced bacteria by about 2 log cycles. Further increases in dose level to 0.5 W s cm^{-2} reduced the bacteria level 3 log cycles. Also, the authors considered that since UV radiation does not penetrate most opaque materials, it was less effective on rough surface cuts of meat because bacteria were partly shielded from the radiation.

Kim et al. (2014) investigated if UV-C light irradiation can be used to improve the safety and storage of Hanwoo beef (Korean native cattle, which provide one of the rarest and expensive meats in the world). The meat samples were exposed to UV radiation (4.5 × 10^{-3} W cm^{-2}) for 0, 5, 10, 15, and 20 min. The UV-C irradiated beef that was exposed for 20 min showed significantly reduced mesophilic and psychrotrophic bacterial populations to the extent of 3 log cycles, as compared to that of non-irradiated beef. Coliform and Gram-negative bacteria were reduced by 1 log cycle. In addition, the authors observed that the population of *L. monocytogenes*, S. Typhimurium, and *E. coli O157*: H7 decreased significantly after UV-C treatment verifying that UV radiation before refrigeration can effectively reduce the number of pathogenic bacteria on the surface of meat.

Likewise, UV-C light can be applied combined with other treatments or technologies to improve its effectiveness. In this sense, the individual effects of UV-C

light (8×10^{-4} W cm^{-2}, during 30 s) radiation and combined with organic acids (lactic acid and peroxyacetic acid), and bacteriophages, on *Salmonella* populations in ground beef was determinate (Yeh et al. 2018). Beef was inoculated with four *Salmonella* strains to result in a contamination level of 3.5 log CFU g^{-1}. Lactic (LA) and peroxyacetic (PAA) acids, bacteriophages (S16 and FO1a) (BA), and ultraviolet light (UV) were applied on fresh trim before grinding. Applications of individual UV light decreased *Salmonella* populations in ground beef by approximately 1 log CFU g^{-1}. When combined, UV and bacteriophage reduced 2 log cycles of *Salmonella* in ground beef.

Degala et al. (2018) studied the individual and combined efficacy of UV-C light and lemongrass essential oil on *E. coli* K12 inoculated in meat. Lemongrass oil at 0.25%, 0.5%, and 1% concentrations with UV-C light intensities of 1×10^{-4} and 2×10^{-4} W cm^{-2}, for 2, 4, 6, 8, 10, and 12 min was applied. The combination of 1% lemongrass EO with UV-C 2×10^{-4} W cm^{-2} (2 min) treatment occasioned a reduction of 6 log CFU mL^{-1} of *E. coli* compared to the control, the level of which was significantly higher than individual and others obstacle treatments.

Although UV interventions are widely used for other foods, for meats there are limitations in its application that must be considered:

1. Oxidation of myoglobin Exposure time: UV Light plays a critical role in pigment photooxidation, since it catalyzes MetMb formation (Djenane et al. 2003) and consequently, detrimental effects on beef color. Oxidation rate depends on time and applied dose.
2. Low UV penetration in food matrices: generally, a strong limiting factor in the efficacy of UV-C decontamination.
3. The existence of the high microbial populations reduces the capacity for UV-C penetration and lower considerably its efficacy.

2.1.2 Organic Acids

The application of organic acids on meat surfaces is a common procedure for longer shelf life. The organics acid treatments are cheap, simple, fast, and have shown clear efficiency antimicrobial. Additionally, organic acids are designated by the FDA as generally recognized as safe (GRAS) for meat products (Mani Lopez et al. 2012).

The antimicrobial activity of organic acids is based on the organic acids have in a pH-dependent equilibrium between the undissociated and dissociated state. The undissociated state of the molecule is principally responsible for the antimicrobial activity. It is believed that the inhibitory action is a result of the compound in the undissociated state being able to freely cross the cell bacterial membrane. Once in the cytoplasm, the molecule dissociates, and the accumulating anions to inhibit metabolic reactions. Other mechanisms that have also been proposed for the inhibition of microbial growth include membrane disruption and stress on intracellular pH homeostasis. Thus, organic acids exhibit both bactericidal and bacteriostatic properties (Jamilah et al. 2008).

There is extensive information in the bibliography on the application of organic acids as surface decontaminant on fresh meat. Among the most used are acetic, citric, lactic, tartaric, gluconic, sorbic and propionic (Coll Cárdenas et al. 2008; Harris et al. 2006; Mohan and Pohlmans 2016; Van Ba et al. 2018; Zhou et al. 2007).

In this sense, Van Ba et al. (2018) determinate significant reductions in numbers of the bacterial populations (2–5 log cycles) such as aerobic plate count, *E. coli*, total coliform, *Staphylococcus, Shigella, Bacillus, Escherichia, Salmonella and Pseudomonas* on the carcass surface beef after spraying with the 3% lactic acid or acetic acid solution. In addition, also demonstrate that the spray with lactic acid generally produced greater reductions in numbers of all the bacterial species as compared to the acetic acid. These acids were also effective in reducing foodborne pathogens in beef trim (Harris et al. 2006). The authors determined that *E. coli* O157:H7 and Salmonella typhimurium were reduced by 1.5–2.0 log cycles when 2% of acids were applied.

Mohan and Pohlmans (2016) studied the antimicrobial efficacy of ten different organic acids (fumaric, malic, citric gluconic, levulinic, pyruvic, caproic, caprylic, and capric acids, at 3%) on inoculated beef to reduce the bacterial population of coliform, *E. coli* O157: H7, and Mesophilic aerobic plate count. The results indicate that caprylic acid was the most effective antimicrobial agent currently approved for use in on beef. On the contrary, gluconic acid tested in this study resulted in no reduction in microorganisms number studied.

One of the limitations of applying organic acids as antimicrobials is that in high concentrations it could cause undesirable sensory changes, in the food that might be taken in consideration. Because of this, combination treatments could achieve the desired antibacterial effect at concentrations low enough to minimize undesirable changes in flavor. In this sense, Zhou et al. (2007) evaluated the synergistic effect of thymol and Carvacrol with acetic acid on Mueller-Hinton broth (MHB) and observed strong synergistic activities.

2.1.3 Essential Oils

Essential oils (EOs) (also called volatile or ethereal oils) are aromatic oily liquids obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots). For commercial production of EOs steam distillation is most used to obtain them. It has long been recognized that some EOs have antibacterial properties (Burt 2007).

The EOs mechanisms of action have not been identified, but they seem to be related to their hydrophobic nature attributed to phenolic compounds, such as the carvacrol, eugenol (2-methoxy-4-(2-propenyl) phenol), and thymol (Sanchez-Gonzalez et al. 2011). In that sense, Chivandi et al. (2016) have determined that their antimicrobial action would be given by mainly altering cell membranes causing loss of cellular constituents, the collapse of membrane structure, and cell death of eukaryotic and prokaryotic microorganisms. Additionally, these oils can impair a

variety of enzyme systems including the enzymes involved in the energy regulation and synthesis of structural components and inactivate or destroy genetic material, strengthening their antimicrobial activities (Burt 2007).

It is important to note that the risk of bacterial resistance is low since essential oils are constituted by mixtures of compounds with various mechanisms of action, which makes it difficult for microorganisms to adapt. In addition, due to their natural origin, they turn out to be safe for consumers, who are increasingly looking for and choosing this type of product.

These oils have been extensively used for centuries in food products. Regarding the meat and meat products, EOs from oregano, rosemary, thyme, clove, balm, ginger, basilica, coriander, marjoram, and basil have shown a greater potential to be used as an antimicrobial agent (Burt 2007; Jayasena and Jo 2013).

The EOs have been shown to possess antibacterial and antifungal activities against several microorganisms associated with meat, including gram-negative and gram-positive bacteria (Jayasena and Jo 2013). In this sense, Tsigarida et al. (2000) reported a reduction in initial microflora by 3 log cycles was detected in beef fillets treated with Oregano EO (0.8%), compared with the level for the control. Hulankova et al. (2013) proved additive effect when combining of 0.2% Oregano EO (v/w) with 0.5% caprylic acid and 0.1% citric acid in vacuum packed minced beef inoculated with *L. monocytogenes* at a concentration of 5 log cells g^{-1}. When compared to control, the combination of Oregano EO, caprylic acid and citric acid decreased counts of lactic acid bacteria by 1.5 log cycles and counts of psychrotrophic bacteria and *L. monocytogenes* by more than 2.5 log cycles at the end of storage (10 days) at 3 °C.

The antimicrobial effect of Rosemary EO combined with modified atmosphere conditions (aerobic, vacuum, or high-O2), to extend the shelf life of beef was confirmed by Sirochi et al. (2017). The authors determined that the use of Rosemary EO proved efficacious in every storage condition, as seen in the lower counts of psychrotrophics, *Brochothrix thermosphacta, Pseudomonas* spp., and Enterobacteriaceae in active packaging meat compared to non-active packaging meat. Particularly, when Rosemary EO was accompanying with high-O2 conditions, the shelf life of meat was extended 8 days longer than meat stored under aerobic conditions.

Mattos de Oliveira et al. (2013) studied the use of Thyme and Rosemary EOs as antibacterial agents for the control of *L. monocytogenes* in raw beef. Two treatment alternatives for applying the mixture of oils were studied: the submission to their vapors (0.74 $\mu L \cdot cm^{-3}$) and the application of edible gelatine coatings (2% v/v). Both options showed an antimicrobial effect, in the experiment that used edible coatings containing EOs, at 48 h of storage reductions in bacterial counts 1.25 log CFU g^{-1} was obtained. Likewise, the vapor effect experiment caused the highest reduction in the population of bacteria inoculated in raw bovine meat 0.40 log CFU g^{-1} at 96 h of storage.

Smaoui et al. (2016) analyzed the antibacterial effect to applied Mentha piperita EO alone (0.25 or 0.5%) or combined with semi-purified bacteriocin BacTN635 (500 or 1000 AU g^{-1}), on minced beef meat. The mayor preservative effect was

achieved by using the combination of Mentha piperita EO at 0.5% with BacTN535 (1000 AU g^{-1}), increasing the shelf life of meat beef by 7 days at 4 °C.

Despite the demonstrated potential of using EOs in meat preservation, some limitations have also been identified in the application as meat preservatives, that need to be overcome before wholesale use. Among which are mainly:

- Due to the possible interaction of EOs with meat constituents, generally a greater concentration of EOs is required to achieve the same effect in meat compared with in vitro assays (Burt 2007).
- Use of high concentrations of the EOs to achieve significant antimicrobial activity causes changes in the sensory quality of the meat, mainly taste and aroma. The strong aroma and flavors associated with some EOs are such that even if low concentrations of the EOs were used to preserve meat, there would be negative organoleptic changes that would affect the acceptability of the preserved meat (Burt 2007).
- It may be difficult to maintain quality consistency because the composition of an individual EO can vary due to several factors including the time of harvesting, variety, and the part of the plant used, and method of extraction (Burt 2007).

To solve these problems, hurdle technology represents an interesting alternative. As previously mentioned, several authors have reported that better results can be obtained by combining lower concentrations of EOs with other antimicrobial compounds and/or other preservative technologies.

2.2 Case Study

As previously mentioned, the short shelf life of chilled fresh meat is known, therefore, it is necessary to explore other alternative technologies that allow its shelf life to be alarmed without altering its sensory quality. Thus, the effect of the combined application of diverse antimicrobial agents: UV-C light, cooling, and Rosemary OE in combination with lactic acid on the growth kinetics spoilage microorganisms in bovine meats surface was studied.

2.2.1 Materials and Methods

2.2.1.1 Physical Agents

The physics agents used in this assay were UV-C light and temperatures of refrigeration.

For irradiation with UV-C light, a piece of equipment was used, designed for this purpose, consisting of two UV-C light tubes (15 watts each and 0.45 m in length, Philips) and shelves located at a distance of 0.15 m in height, Fig. 2.1.

Fig. 2.1 Equipment designed for irradiated of meat samples with UVC light. In the top are the UVC light tubes

In this work were used two bovine meat cuts of different pHs. On the one hand, the cut known as buttock (*Biceps femoris* muscle) of pH 5.6 and on the other hand, beef, (*Longissimus dorsi* muscle) of pH 5.8. The pH values were determined using a Termo-peachimeter of punction Meter 6171L.

And concerning the cooling temperatures, three temperature-controlled chambers were used at 0, 4 and 8 °C, where the meat samples were stored for the duration of the experience.

2.2.1.2 Natural Additives

For this work was used EO of Rosemary (*Rosmarinus officinalis L.*) with a density of 800 kg m^{-3}, purchased in the local market (Beatriz cosmetics S.R.L). Were used dilutions of EO in propylenglycol (Anedra Research AG S.A.), employed that solvent and combined with lactic acid (Parafarm) (1:1). MIC of mixture EO and lactic acid was determined previously in correlation with results obtained by Talero (2019).

2.2.1.3 Studies in Meat

Meat samples were obtained from Biceps femoris and Longissimus dorsi muscles (pH 5.6 and 5.8 respectively), from steers, carcass weighing up to 240 kg, with a post-mortem time of 48 h at 4 °C, purchased locally and analyzed separately. Then, the samples were treated aseptically and were cut into circular samples of 19.6×10^{-4} m^2 with a sterile scalpel (approximately 0.01 kg of meat sample). After, the samples ($n = 60$), were placed in sterile Petri dishes for their subsequent treat with UV-C light and EO. The irradiation with UV-C light was for 5 min. The total irradiation dose received by the samples was 5.40×10^{-3} kJ m^{-2} determined by a

digital radiometer (Cole Parmer) (Fernández Blanco et al. 2014, 2017). Then these were sprayed with 1 mL of MIC of mixture EO and lactic acid (1:1). Samples untreated were considered as Control. The samples were separated into three sets and stored in cold storage with controlled temperature at 0, 4 and 8 °C for 2 weeks.

The experiences were carried out in quadruplicate.

2.2.1.4 Microbiological Analysis

At different storage times (0–15 days), the surface samples were swathed with sterile swabs and placed in 9 mL sterile 0.1% peptone broth. Decimal dilutions with peptone water were then performed. For Total Aerobic Microorganisms enumeration, 1 mL of serial dilution were placed in Plate Count Agar, *Pseudomonas* sp. in Cetrimide Agar and for Enterobacteriaceae, Crystal Violet, Neutral Red Bile Agar; the Plate Pour Procedure was used with aerobic incubation at 37 °C for 48 h for all cases. Those bacteria were studied for being the most found in meats.

Determinations were made in duplicate. For all results, an Ionomex colony counter was used to quantify the results and the counts were expressed as log N (N: Colony Forming Units/cm^2 (CFU cm^{-2})).

2.2.1.5 Mathematical Modeling

One of the most recommended models is the Gompertz modified equation (Coll Cárdenas et al. 2008). From this equation, the following derived parameters were obtained: specific growth rate, $\mu = b \,.\, c/e$ [log (CFU cm^{-2}) days^{-1}], with $e = 2.71$; lag phase duration, LPD = $m - (1/b)$ [days]; maximum population density, MPD = $a + c$ [log (CFU cm^{-2})]. Data fits obtained from Gompertz model were analyzed by means of Systat software (Systat Inc.12.0). It calculates the set of parameters with the lowest residual sum of squares and their 95% confidence interval. Statistical analysis of experimental measurements (analysis of variance and pair wise comparisons) was computed using the same software. Differences in means and F-tests were considered only when $p < 0.05$.

2.2.2 Results and Discussion

First, it should be noted that according to results was observed that each bacterium presented a different sensibility for each treatment.

In theory, using mixtures of antimicrobial agents provides a wider range of activity, which increases antimicrobial activity against pathogenic or deteriorative microorganisms. It is believed that a mixture of antimicrobial agents can act upon different microbial species, or act on several vital points (or targets) inside the cells of similar microbial species, which can enable better control of the microorganisms in food,

compared to the use of an individual antimicrobial agent (Santiesteban-Lopez et al. 2007; Garcia-Garcia et al. 2011). These concepts would support the results observed.

Figure 2.2 shows the microbial growth of Total Aerobic Microorganisms, *Pseudomonas* sp., and Enterobacteriaceae in *Longissimus dorsi* (a, b, c) and *Biceps femoris* muscles (d, e, f) after irradiated with UV-C light and sprayed with a mixture EO and lactic acid (1:1) at 0 °C storage. Control samples untreated were also analyzed. The figure shows the application of the Gompertz and linear regression models. A good agreement was observed between the model and the experimental data; the obtained parameters are shown in Table 2.2. Microbial growth in treated and untreated samples showed significant differences ($p < 0.05$).

Analyzed the results observed in Fig. 2.2, it can be seen in all cases that samples treated showed the minors, final counts, during the times of the experience (2 weeks). The highest values of final counts were presented for Enterobacteriaceae in Control samples (Fig. 2.2c, f) for *Longissimus dorsi* and *Biceps femoris* muscles (2.62 and 2.43 log CFU cm^{-2}, respectively), which is observed a difference of 0.83 and 2.43 log cycles with the same family in corresponding treated one.

In both muscles, the microbial counts of Total Aerobic Microorganisms had to be modeled by linear regression due to the poor development that these microorganisms presented, observing in all cases a difference <0.5 log CFU cm^{-2} between the final and initial counts.

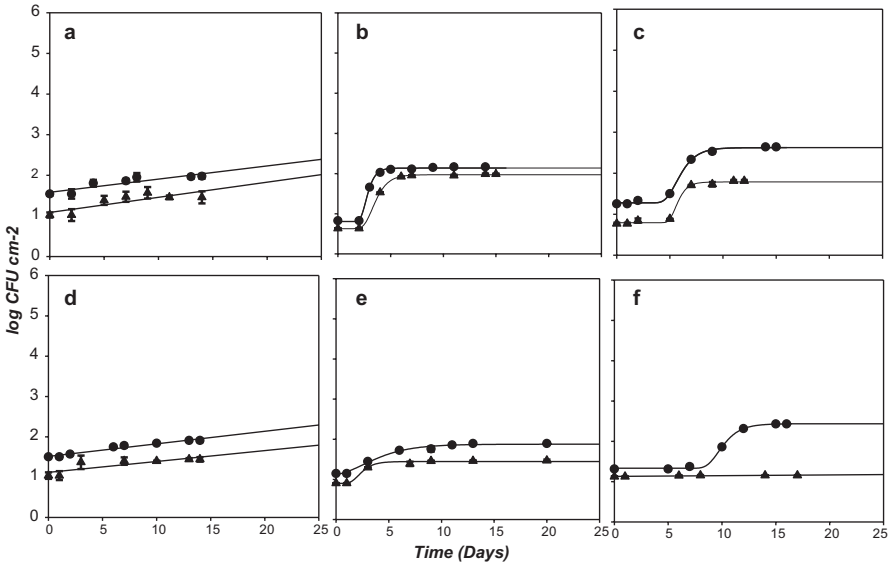

Fig. 2.2 Development of Total Aerobic Microorganisms, *Pseudomonas sp* and Enterobacteriaceae in *Longissimus dorsi* (**a**, **b**, **c**, respectively) and *Biceps femoris* muscles (**d**, **e**, **f**, respectively) at 0 °C storage. Treated (▲) and Control samples (•) with error bars. Full lines correspond to Gompertz or linear models

Table 2.2 Mathematical modeling of microbial growth in meat samples storage at 0 °C

	Gompertz parameters				Derivated parameters		
	a	*c*	*b*	*m*	*μ*	LPD	MPD
	Longissimus dorsi						
	Total aerobic microorganisms						
Control samples	—	—	—	—	0.032	15.62	$R^2$0.81
Treated samples	—	—	—	—	0.030	16.66	$R^2$0.70
	Pseudomonas sp						
Control samples	0.807 ± 0.030	1.3304 ± 0.0351	2.032 ± 0.269	2.620 ± 0.065	0.994	2.128	2.137
Treated samples	0.629 ± 0.014	1.344 ± 0.017	1.231 ± 0.139	3.256 ± 0.093	0.608	2.443	1.973
	Enterobacteriaceae						
Control samples	1.278 ± 0.024	1.346 ± 0.037	1.029 ± 0.109	5.594 ± 0.106	0.509	3.841	2.625
Treated samples	0.795 ± 0.025	0.993 ± 0.037	0.602 ± 0.125	5.523 ± 0.149	0.220	3.864	1.788
	Biceps femoris						
	Total aerobic microorganisms						
Control samples	—	—	—	—	0.031	16.12	$R^2$0.95
Treated samples	—	—	—	—	0.027	18.51	$R^2$0.70
	Pseudomonas sp						
Control samples	1.084 ± 0.007	0.793 ± 0.116	2.345 ± 0.651	2.641 ± 0.729	0.685	0.426	1.878
Treated samples	0.903 ± 0.036	0.542 ± 0.041	0.759 ± 0.514	2.130 ± 0.634	0.151	0.814	1.445
	Enterobacteriaceae						
Control samples	1.333 ± 0.016	1.103 ± 0.029	1.091 ± 0.195	9.669 ± 0.108	0.443	8.753	2.437
Treated samples	—	—	—	—	0.001	>25	$R^2$0.74

a: [log (CFU cm^{-2})]; *c*: [log (CFU cm^{-2})]; *b*: [log (CFU cm^{-2} days^{-1})]; *m*: [days]; *μ*: [log (CFU cm^{-2}) days^{-1}] LPD: [days]; MPD: [log (CFU cm^{-2})]

Although each growth curve presented particular kinetics, only in the case of Enterobacteria a greater influence of the pH was observed, since for the treated samples of *Biceps femoris* (pH 5.6) the linear regression model should be used to analyze its representation.

In Table 2.2 presents the Gompertz and Derived Parameters then to model mathematically the microbial growth in all sample's storage at 0 °C.

To analyze these results, it was observed that *Pseudomonas* sp. in Control samples shows the most values of μ for both muscles (0.99 and 0.68 log CFU cm^{-2} days^{-1}),

being 4.5 and 1.6 times higher than in the samples treated for *Longissimus dorsi* and *Biceps femoris*, respectively.

Concerning the LPD parameter, untreated samples of Enterobacteriaceae presented the highest values (>25 days), while the lowest values were presented by *Pseudomonas* sp. (0.42–0.81 days), demonstrating a very good development at this temperature. In this sense, Castaño et al. (2010), informed that the ethanolic extract and leaf essential oil from *Rosmarinus officinalis* L. showed a broad spectrum of antimicrobial action for both Gram-positive and Gram-negative bacteria of interest in the food industry.

Figure 2.3 shows the microbial growth of meat samples for both muscles with and without treatment, storage at 4 °C during the days that the experience lasted.

When analyzing Fig. 2.3 it was observed that the highest microbial growth values were found in the control samples of *Longissimus dorsi* (4.42, 3.41 and 4.51 log CFU cm^{-2} for Total Aerobic Microorganisms, *Pseudomonas* sp and Enterobacteriaceae, respectively).

Only in the case of the treated samples of *Biceps femoris*, for the counts of *Pseudomonas* sp, should the linear regression model be used for its representation (Fig. 2.3e). The greatest differences between the final counts of the treated and untreated samples were presented for this muscle (1.79 and 2.52 log cycles for *Pseudomonas* sp and Enterobacteriaceae respectively).

In Table 2.3 was observed the Gompertz and Derived Parameters then to model mathematically the microbial growth in all sample's storage at 4 °C.

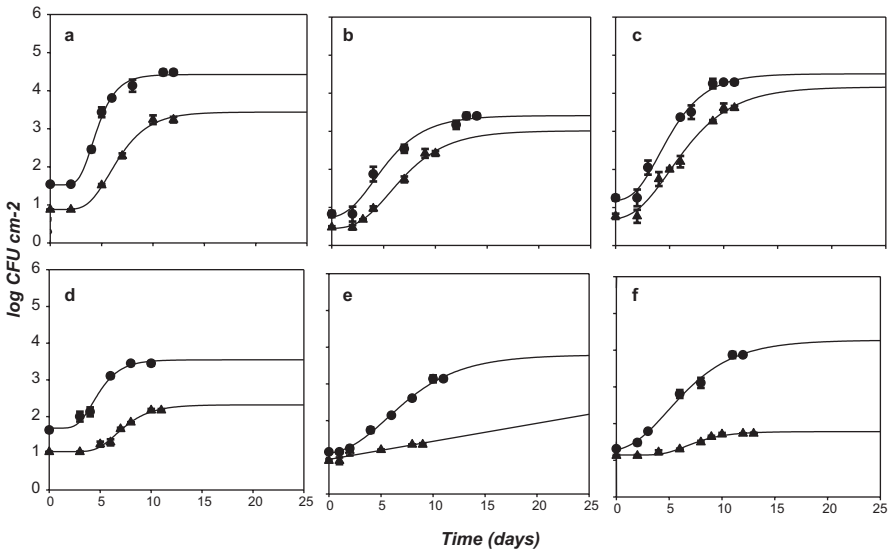

Fig. 2.3 Development of Total Aerobic Microorganisms, *Pseudomonas sp* and Enterobacteriaceae in *Longissimus dorsi* (**a**, **b**, **c**, respectively) and *Biceps femoris* muscles (**d**, **e**, **f**, respectively) at 4 °C storage. Treated (▲) and Control samples (•) with error bars. Full lines correspond to Gompertz or linear models

Based on the observed results in Table 2.3, could be determined that the highest values of μ corresponded to the Control samples of *Longissimus dorsi* for Total Aerobic Microorganisms (0.83 log CFU cm^{-2} $days^{-1}$).

Regarding the LPD, *Pseudomonas* sp in the treated samples of *Biceps femoris* presented the longest period (10 days), observing the greatest difference regarding this parameter of the control samples, untreated (8.43 days).

Table 2.3 Mathematical modeling of microbial growth in meat samples storage at 4 °C

	Gompertz parameters				Derivated parameters		
	a	*c*	*b*	*m*	*μ*	LPD	MPD
	Longissimus dorsi						
	Total aerobic microorganisms						
Control samples	1.518 ± 0.089	2.898 ± 2.898	0.779 ± 0.127	4.081 ± 0.150	0.831	2.798	4.426
Treated samples	0.879 ± 0.079	2.559 ± 0.200	0.480 ± 0.113	5.761 ± 0.272	0.827	3.680	3.438
	Pseudomonas sp						
Control samples	0.717 ± 0.244	2.695 ± 0.347	0.376 ± 0.124	4.075 ± 0.656	0.373	1.418	3.412
Treated samples	0,443 ± 0,101	2571 ± 0,507	0,332 ± 0,099	5602 ± 0,717	0.314	2.598	3.014
	Enterobacteriaceae						
Control samples	1.170 ± 0.202	3.340 ± 0.349	0.426 ± 0.095	4.056 ± 0.449	0.524	1.713	4.510
Treated samples	0.670 ± 0.211	3.494 ± 0.672	0.312 ± 0.111	4.992 ± 0.554	0.402	1.796	4.165
	Biceps femoris						
	Total aerobic microorganisms						
Control samples	1.686 ± 0.143	1.859 ± 0.233	0.676 ± 0.219	4.256 ± 0.385	0.462	2.778	3.546
Treated samples	1.047 ± 0.045	1.273 ± 0.138	0.526 ± 0.128	6.541 ± 0.283	0.246	4.641	2.320
	Pseudomonas sp						
Control samples	1.072 ± 0.114	2.718 ± 0.495	0.258 ± 0.071	5.617 ± 0.588	0.258	1.743	3.791
Treated samples	—	—	—	—	0.049	10.18	$R^2$0.85
	Enterobacteriaceae						
Control samples	1.231 ± 0.185	3.040 ± 0.428	0.285 ± 0.071	4.887 ± 0.539	0.319	1.388	4.272
Treated samples	1.144 ± 0.026	0.637 ± 0.061	0.476 ± 0.121	6.555 ± 0.367	0.111	4.457	1.781

a: [log (CFU cm^{-2})]; *c*: [log (CFU cm^{-2})]; *b*: [log (CFU cm^{-2} $days^{-1}$)]; *m*: [days]; *μ*: [log (CFU cm^{-2}) $days^{-1}$] LPD: [days]; MPD: [log (CFU cm^{-2})]

These control samples could not be modeled with the Gompertz model, and the linear regression model must be used. A good coefficient of determination R^2 was observed for this parameter (0.85).

Figure 2.4 shows the microbial development of the treated and untreated samples with the same conditions but stored at a higher temperature (8 °C), during the time the experience lasted.

All curves presented in Fig. 2.4 could be modeled from the Gompertz model. At this storage temperature, the highest final counts were observed corresponded to the untreated samples of *Longissimus dorsi* (4.69 log CFU cm^{-2}). While for *Biceps femoris*, the greatest differences between the final counts of the untreated and treated samples were presented (between 1.65 and 1.88 log CFU cm^{-2}).

As in the other storage temperatures, Table 2.4 shows the mathematical modeling of microbial growth at these temperatures.

When storing the samples at this temperature, a very good microbial development was observed, presenting high values of μ, between 0.34 and 0.75 log CFU cm^{-2} $days^{-1}$, and very few differences between the values of this parameter between the samples treated and untreated (0.01–0.31 log CFU cm^{-2} $days^{-1}$).

The greatest differences of LPD between the treated and untreated samples were presented for Enterobaceriaceae, of both muscles (3.05 days and 2.04 days for *Longissimuns dorsi* and *Biceps femoris*, respectively), showing the difference in

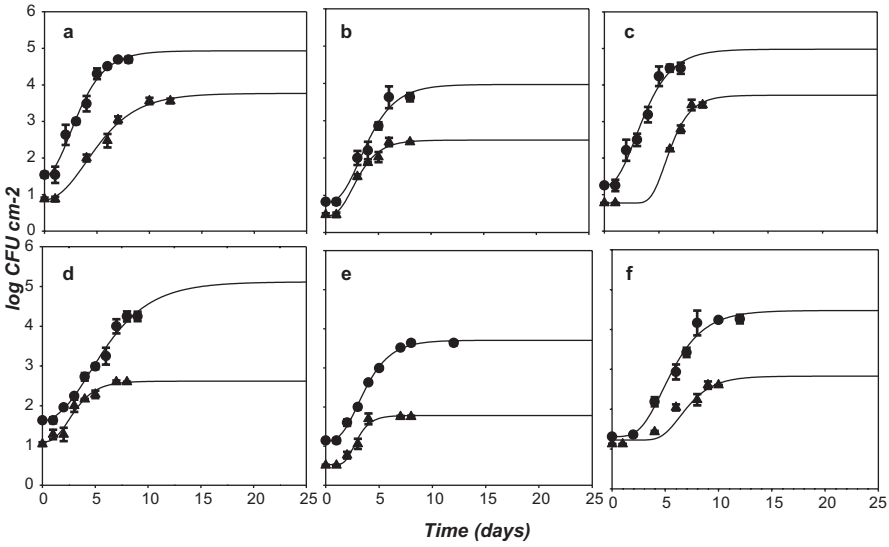

Fig. 2.4 Development of Total Aerobic Microorganisms, *Pseudomonas sp* and Enterobacteriaceae in *Longissimus dorsi* (**a**, **b**, **c**, respectively) and *Biceps femoris* muscles (**d**, **e**, **f**, respectively) at 8 °C storage. Treated (▲) and Control samples (•) with error bars. Full lines correspond to Gompertz or linear models

Table 2.4 Mathematical modeling of microbial growth in meat samples storage at 8 °C

	Gompertz parameters				Derivated parameters		
	a	*c*	*b*	*m*	μ	LPD	MPD
	Longissimus dorsi						
	Total aerobic microorganisms						
Control samples	1.398 ± 0.298	3.534 ± 0.487	0.553 ± 0.149	2.475 ± 0.353	0.719	0.669	4.932
Treated samples	0.8022 ± 0.196	2.964 ± 0.371	0.374 ± 0.499	3.971 ± 0.499	0.408	1.303	3.768
	Pseudomonas sp						
Control samples	0.782 ± 0.259	3.190 ± 0.657	0.542 ± 0.252	3.227 ± 0.466	0.637	1.220	3.972
Treated samples	0.431 ± 0.119	2.048 ± 0.202	0.717 ± 0.192	2.523 ± 0.299	0.540	1.128	2.480
	Enterobacteriaceae						
Control samples	1.191 ± 0.306	3.788 ± 0.760	0.555 ± 0.214	2.889 ± 0.413	0.773	1.088	4.079
Treated samples	0.770 ± 0.096	2940 ± 0.361	0.700 ± 0.367	5.571 ± 0.225	0.757	4.142	3.710
	Biceps femoris						
	Total aerobic microorganisms						
Control samples	1.558 ± 0.194	3558 ± 0.779	0.323 ± 0.104	4.550 ± 0.567	0.423	1.160	5.111
Treated samples	1.081 ± 0.137	1538 ± 0.212	0.718 ± 0.242	2.502 ± 0.368	0.406	1.111	2.619
	Pseudomonas sp						
Control samples	1.121 ± 0.062	2.586 ± 0.092	0.606 ± 0.054	3.027 ± 0.116	0.577	1.379	3.708
Treated samples	0.527 ± 0.088	1256 ± 0.129	1.176 ± 0.392	2.619 ± 0.245	0.544	1.769	1.784
	Enterobacteriaceae						
Control samples	1.310 ± 0.178	3.166 ± 0.335	0.468 ± 0.121	4.755 ± 0.410	0.545	2.618	4.478
Treated samples	1.223 ± 0.092	1.603 ± 0.721	0.580 ± 0.845	6.383 ± 0.668	0.342	4.660	2.830

a: [log (CFU cm^{-2})]; *c*: [log (CFU cm^{-2})]; *b*: [log (CFU cm^{-2} $days^{-1}$)]; *m*: [days]; μ: [log (CFU cm^{-2}) $days^{-1}$]; LPD: [days]; MPD: [log (CFU cm^{-2})]

behavior against the treatment of different groups of bacteria (Radha Krishnan et al. 2015).

In all cases, a good fit of the experimental data to the models is observed ($R^2 > 0.85$).

Results presented in this chapter demonstrate that the action of different antimicrobial agents (physical and natural), used together are effective in reducing the superficial microbial flora of meats.

Several studies have proven the effectiveness of the use of combined methods as obstacles to prevent the development of contaminating microbial agents, knowing that the application of these methods individually fails to provide adequate conservation (Tassou et al. 2000). Jayasena and Jo (2013); Rivera et al. (2014); Huq et al. (2015) and Talero (2019) guarantee our experiences observing a greater action of these agents when they are used in combination with other methods, be it a mixture of oils, other acids such as lactic, maleic, different temperatures, etc., producing in most cases synergistic effects such as decontaminants, without altering the quality of different foods.

2.3 Conclusions

Throughout this chapter it has been observed that in all cases control samples showed a higher bacterial count than those treated, demonstrating the antimicrobial action of the different study agents used together.

Although these technologies have shown very good antimicrobial activity, their use and application in the food industry have been limited because each one individually presents some limitation, oxidation in the case of UV-C light; intense aroma in the case of EOs, so we believe that the use of these technologies would be more efficient if they are applied in combination to improve microbial stability and sensory quality.

Moreover, Gompertz derived parameters were a useful tool for differences in the effect of treatments on the different groups of microorganisms studied in the present work.

Based on the foregoing, we believe that the application of these technologies can be a promising alternative to the use of chemicals to extend the shelf life of food.

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Chapter 3
Effects of Depuration on Subsequent Deterioration and Shelf Life of Cultured Grooved Carpet Shell Clam *Ruditapes decussatus* During Chilled Storage

Fábio Mota, Jaime Aníbal, and Eduardo Esteves

3.1 Introduction

The grooved carpet shell clam *Ruditapes decussatus* (L., 1758), which occurs from the eastern Atlantic to the Mediterranean, is one of the most consumed and profitable mollusks in the Mediterranean (Aníbal et al. 2011; FAO 2020). In Portugal, ca. 88% of the production is originated in the Ria Formosa (INE/DGRM 2019), a highly productive, 18.500 ha coastal lagoon system limited by a streak of barrier islands located in southern Portugal (36°58′N, 8°02′W to 37°03′N, 7°32′W) (Almeida and Soares 2012). *R. decussatus*' nutritional value, namely its high protein content, and very low level of fat, <1 g/100 g, and cholesterol (ca. 45 mg/100 g) make it a valuable seafood product. Condition and nutritional value (related to protein content) of clams in the Ria Formosa is higher in early-Summer (May–June) (Aníbal et al. 2011). Notwithstanding, like other filter-feeding species, there are risks associated with consumption derived from their contamination with chemicals (El-Shenawy 2004), microorganisms (Almeida and Soares 2012), or biotoxins

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(O'Mahony 2018; Vale et al. 2008). Presently, the classification system for the bivalves' production areas is based on bacteriological counts (*E. coli*) and heavy metals' contents (cadmium, lead and mercury) (EU 2004a, b, 2005a, 2006, 2007, 2008a, 2015; Ministério da Agricultura, do Desenvolvimento Rural e das Pescas 2006). Depending on the water quality of their environment, specimens have to be depurated, a remediation technique, before marketing (EU 2005a). Similar requirements and measures apply in the USA (US FDA 2009). Oliveira et al. (2011) provide an updated overview of this topic.

The process of depuration consists in maintaining the clams in cold and clean, sterile seawater for 24–48 h to eliminate or significantly reduce the bacterial load that was eventually accumulated during growth in production areas (Lee et al. 2008; Maffei et al. 2009; Ruano et al. 2012). Parameters such as shellfish suitability (in terms of salubrity and condition after harvesting and general handling), physiological conditions (*viz*. dissolved oxygen, loading, shellfish to water ratio, water flow, salinity, temperature, turbidity and disturbances), and infrastructures and operations (design of the operating system, basic hygiene draining, batch control, water quality) are critical (Lees et al. 2010). Clams are marketed live, packed in plastic net bags and stored at chill temperatures (ca. 5 °C) (Ministérios da Economia e da Inovação e da Agricultura, do Desenvolvimento Rural e das Pescas 2006). Their post-mortem spoilage dynamics is dependent on the concentration of substrates and metabolites, endogenous enzymes activity and microbial contamination (Sikorski and Kołakowski 2011) and is still also poorly understood (Anacleto et al. 2015). Commonly, parameters such as the survival of specimens, condition index (CI), glycogen content, pH, total volatile basic nitrogen (TVB-N) and trimethylamine nitrogen (TMA-N) contents, total viable counts (TVC), and/or abundances of *Escherichia coli*, coliforms, Enterobacteriaceae and psychrotrophic bacteria have been used to assess health status, spoilage dynamics and safety of bivalves subjected to distinct processing technologies and stored under diverse conditions. It is a common belief among clam farmers that depuration decreases appreciably the condition and quality of the individuals, lowering their marketability and economic revenue.

This study aimed to examine the effects of depuration on parameters of biological (mortality rate), physiological/commercial (condition index and percent edibility), physicochemical (pH and TVB-N content), microbiological (TVC, Enterobacteriaceae and psychrotrophic bacteria) and sensory quality of commercial-size clams stored at chill temperatures.

3.2 Material and Methods

3.2.1 Sampling

Samples of *R. decussatus* clams were obtained directly from a local licensed producer/certified depuration center. This ensured that all tested biological material was of commercial value and from the Ria Formosa, Algarve. Within 2–3 h after harvest or the agreed period of depuration (see below), the samples were transported to the laboratory in a refrigerated box and washed before experiments or analyses. At the start of each trial, a subset of clams was randomly selected for biometric measurements and for the determination of the percent edibility and condition index (see below).

3.2.2 Survival of Clams Kept in Refrigeration

The dynamics of survival of non-depurated clams were studied during the winter (December/January) and the late spring (May/June). Clams, n(winter) = 50 and n(summer) = 30, were kept under refrigerated conditions (5 ± 1 °C) for up to 30 days. Daily, specimens were individually and gently stimulated with a stiletto and the behavioral response noted as 1-fast valve reaction, 2-slow valve reaction; 3-no reaction but closed valves; and 4-no reaction and valves open. The number of specimens per day classified as 4 was used to estimate mortality rates and time required for 50% mortality (i.e. median time to death) (see below).

3.2.3 Effects of Storage Temperature on Post-Mortem Changes in pH and TVB-N Content

A sample (n = 194) of non-depurated clams were frozen at −18 °C beforehand to assure the post-mortem status to all specimens in the sample simultaneously. At the beginning of the trial, the clams were thawed at ambient temperature (ca. 20 °C) for 1 h and divided randomly into three groups. Each group was then maintained at 5, 15 and 25 °C. pH and TVB-N content were measured at regular intervals until the obvious deterioration of the samples in terms of sensory and chemical parameters.

3.2.4 *Effect of Depuration on Survival, Condition and Edibility, pH, TVB-N Content, Microbiological and Sensory Quality*

This experimental trial was conducted using n = 720 clams obtained in late Spring (during the season of peak consumption). Half of the specimens were depurated for 24 h in recirculated tanks filled with UV sterilized water at 14 °C at a licensed center in Olhão under customary commercial conditions employed in local facilities, while the other half was transported directly to laboratory in insulated box(es). In the lab, clams were stored packed in closed plastic net bags at chill temperatures (5 ± 1 °C) for up to 24 days.

At the start and at regular intervals, specimens were sampled to estimate the mortality (as in Sect. 3.2.2 above), condition index and percent edibility, pH, TVB-N content, microbiota abundance (TVC, Enterobacteriaceae and psychrotrophic bacteria counts) and sensory quality.

3.2.5 *Quality Parameters*

3.2.5.1 Condition Index and Percent Edibility

At the start of each experiment, individual clams were weighed (±0.1 mg) and their maximum length measured (to 0.05 mm) using a precision caliper. Clams were manually shucked by cutting the adductor muscle with a knife, and the meat was pressed with blotting paper to remove excess moisture before weighting (wet weight, WW, ±0.1 mg). The meat and shells were subsequently dried at 65 °C for 24 h and weighed again to obtain the dry weight (DW, ±0.1 mg). Condition index (CI) was calculated as

$$\mathrm{CI} = \left(\mathrm{MDW} / \mathrm{SDW}\right) \times 1000$$

where MDW is meat dry weight (g) and SDW is the shell dry weight (g) (Aníbal et al. 2011; Orban et al. 2011). Percent edibility (PE) was calculated as

$$\mathrm{PE} = \left(\mathrm{MWW} / \mathrm{TW}\right) \times 100\%$$

where MWW is meat wet weight (g), and TW is the total clam weight including the shell (g) (Aníbal et al. 2011; Mohite et al. 2008; Orban et al. 2011).

3.2.5.2 pH and TVB-N

The pH was measured directly in the meat of individual specimens using an appropriate probe connected to a pH meter (GLP21, Crison®, Spain). The TVB-N content was determined following the micro diffusion method of Conway as described in IPQ (2009) and instructed in the EU Regulation no. 2074/2005 (EU 2005b). Triplicate samples (10 g), each consisting of ca. 10 specimens were used for the assessment of TVB-N content.

3.2.5.3 Microbiological Parameters

Sample preparation was carried out following international standard practice (ISO 2003). In short, samples (20 g) of clams' meat were aseptically placed into sterile Stomacher® bags containing 90 ml of peptone water with NaCl (0.85% w/v) (Merck, Darmstadt, Germany) and homogenized for 1 min (Stomacher 400, Seward Ltd., London, UK). Aliquots of 1 ml were poured in Petri dishes according to serial decimal dilutions before the addition of appropriate media. For the enumeration of mesophilic aerobic (TVC) and psychrotrophic bacteria, plate count agar (PCA, Scharlau, Germany) was incubated at 30 °C for 72 ± 3 h (IPQ 2002) and at 6.5 °C for 10 days (ISO 2001), respectively. Enterobacteriaceae were enumerated after the inoculation of 1 ml aliquots into 10 ml of molten (at 45 °C) violet red bile glucose agar (VRBGA, Scharlau, Germany). After settling, a 10 ml overlay of molten media was added, and VRBGA plates were incubated at 37 °C for 24 ± 1 h (IPQ 1991). All plates were examined visually for typical colony types and morphological characteristics associated with each medium. Microbiological data, i.e. the number of colony-forming units (cfu) per unit mass were log-transformed prior to analysis, as log(cfu)/g.

3.2.5.4 Sensory Analysis

Sensory evaluation of raw and cooked clams was conducted on days 0, 1, 2, 3, 7, 11 and 14 during the storage at 5 °C using a panel of 11 individuals co-opted from the faculty, staff and graduate students of the Departamento de Engenharia Alimentar, Instituto Superior de Engenharia da Universidade do Algarve, that are experienced in the sensory assessment of seafood products.

Each panelist assessed seven sensory attributes of appearance, color (surface brightness, cream-ivory color, white/milky color and yellow/brownish color) and odor (intensity of fresh, ammoniac, and sulfide/putrid odors) of raw and cooked specimens using a 6-point scale (from 0, absent, to 5, extremely intense) that was adapted from Gonçalves et al. (2009) and Meilgaard et al. (2007). The panelists were also asked if at that time they would consume the samples or not. Raw clams were simply shucked before analysis whereas in the case of sensory analysis of

cooked clams, specimens were steamed in a microwave oven for 1 min (at 400 W) before being served to panelists.

3.2.6 Statistical Analysis

Results are given as mean ± standard deviation (descriptive statistics) or parameter estimates ± standard error (regression models). Comparisons of PE and CI among sampling dates in depurated and non-depurated clams were carried out using one-way ANOVA. Mortality (p) over storage time (t, in days) was modeled via logistic regression and the median time to death t_{50}, i.e. the time at which 50% of the specimens are dead, derived from the best fit model. The following form of the two-parameter logistic model was fitted,

$$p = \frac{1}{\left[1 + \exp^{\left(-r\left(t - t_{50}\right)\right)}\right]} \tag{3.1}$$

where p is the proportion of specimens alive, r is the rate (d^{-1}) and t_{50} is the median time to death (d). Dynamics of TVB-N content (y, mg N/100 g) over time (t, in days) was modeled using a first-order, exponential model,

$$y = a\exp\left(r\,t\right) + c \tag{3.2}$$

where a is the initial value, r is the rate (d^{-1}), and c is TVB-N content at t = 0. Clams' acceptability (measured as the proportion of total panelists willing to consume the clams at given day) was modeled using a modified version of the two-parameter logistic model common in psychometric research,

$$p = \frac{1}{\left[1 + \exp^{\left(-k\left(t - t_{50}\right)\right)}\right]} \tag{3.3}$$

that allowed the estimation of the median time to rejection, t_{50}. The exponential and the two-parameter logistic models were fitted via nonlinear regression analysis (using the Levenberg-Marquardt algorithm thru the function nls in R) and their goodness-of-fit assessed using the residual deviance and pseudo-R^2 (Ritz and Streibig 2009). All statistical procedures were carried out at the 0.05 level of significance and using R statistical software (R Core Team 2019).

3.3 Results and Discussion

3.3.1 Survival of Non-depurated Clams

The logistic model fitted the data on proportion alive over storage time well (pseudo-R^2 = 0.996 for Summer and pseudo-R^2 = 0.988 for Winter). The survival of non-depurated clams kept in refrigeration was substantially lower in Summer compared to Winter (Fig. 3.1). The median time to death, t_{50}, was 12.1 ± 0.05 d compared to 20.1 ± 0.22 d, respectively.

In a relatively similar experiment, Anacleto et al. (2013) kept non-depurated clams *Venerupis pullastra* and *Ruditapes philippinarum* at 4 °C and registered their mortality. These specimens' t_{50} were observed to be 5 and 14 days, respectively for *V. pullastra* and *R. philippinarum*. Marin et al. (2005) studied the effects of mechanical stress on under-sized *T. (Ruditapes) philippinarum* survival. Partly, the differences found in t_{50} among studies relate to methods of appraisal of clams' living. Moreover, the temperature difference between their environment/habitat and the refrigerating temperature is much more pronounced in Summer (commonly >20 °C vs. 5 ° C) than in Winter (~15 °C vs. 5 °C). Possibly, the greater thermal shock in Summer stresses animals considerably more such that survival is shortened.

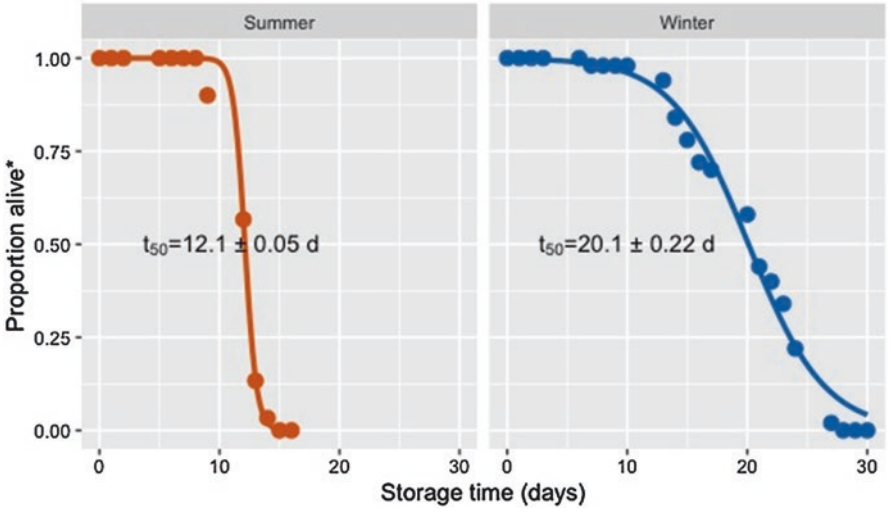

Fig. 3.1 Survival of non-depurated *R. decussatus* clams collected in Summer and Winter when in chilled storage. Proportion alive* (i.e. animals except those where shell gape occurred and external stimuli did not produce any response) and t_{50} is the median time to death (d) (n = 50 and 30 per season)

3.3.2 *Post-Mortem Changes*

The TVB-N content of non-depurated clams maintained at 5, 15 and 25 °C post-mortem (i.e. frozen at −18 °C before the experiment) were relatively low at the start of the trial (3.45 to 5.54 mg N/100 g) and increased exponentially with storage time, but at different rates (Fig. 3.2a). The rates increase from 0.31 ± 0.06 d^{-1} at 5 °C, to 0.72 ± 0.17 d^{-1} at 15 °C to 1.60 ± 0.22 d^{-1} at 25 °C. Considering the limits stipulated in the EU (EU 2005b, 2008b) (albeit for fish), the TVB-N content is estimated to exceed 35 mg N/100 g after 7, 2 and only 1 d, respectively for 5, 15 and 25 °C storage temperatures.

TVB-N concentrations in fresh fish are expected to be non-zero since ammonia is a metabolite already present (Pereira and Tenuta-Filho 2005). The same is anticipated for other seafood. Initial values found herein are in line with values reported by Cao et al. (2009) for Pacific oysters (*Crassostrea gigas*), 4.25 mg N/100 g, but

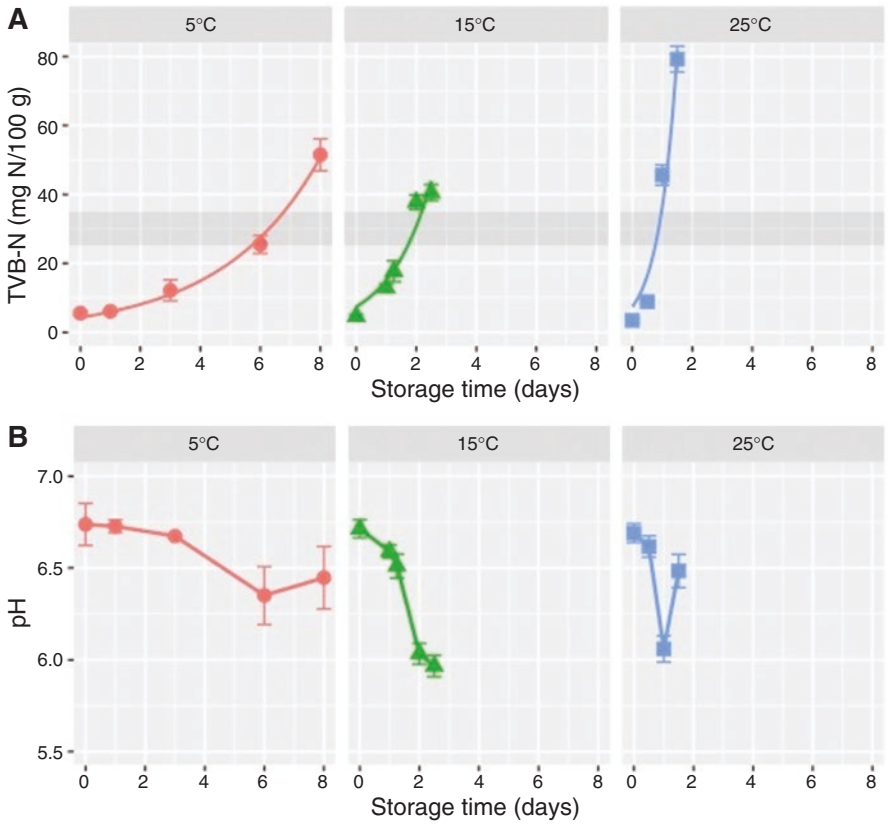

Fig. 3.2 Post-mortem changes in (**a**) TVB-N content and (**b**) pH in non-depurated, natural clams *R. decussatus* during storage at three distinct temperatures, 5, 15 and 25 °C (mean ± SD, n = 150 per treatment)

are substantially lower those reported by Rey et al. (2012) for clams (*V. rhomboideus*), 24 mg N/100 g, and for oyster (*Ostrea edulis*), 12–13.6 mg N/100 g, and by Tosun et al. (2018), Rey et al. (2012), Caglak et al. (2008), and Manousaridis et al. (2005) for raw mussels *Mytilus galloprovincialis*, 17.52 mg N/100 g, 18.99 mg N/100 g, 11.48 mg N/100 g and ca. 10 mg N/100 g, respectively. Values observed at the start of our trial are indicative of clams' freshness. Despite the low levels of non-protein nitrogenous compounds in bivalves, the production of volatile compounds resulting from the post-mortem degradation usually determines the increase in TVB-N content. Kim et al. (2002) recorded TVB-N values between 19 and 35 mg N/100 g for oysters packaged in LDPE pouches after 12 days of refrigerated storage. Manousaridis et al. (2005) reported increasing values of TVB-N content for control (vacuum-packaged) samples of shucked mussels up to 31.9 mg N/100 g after 12 days of storage at 5 °C. Similarly, Caglak et al. (2008) found rapidly increasing concentrations of TVB-N in the air- and vacuum-packaged shucked mussels up to 64 mg N/100 g (after 8 d) and 66.6 mg N/100 g (after 12 d). Herein, post-mortem concentration of TVB-N in clams stored refrigerated, at 5 °C, showed similar trends.

Although in pasteurized samples of marinated mussels *M. galloprovianclis* stored for 21 d at 4 °C, Tosun et al. (2018) observed increasing concentrations of TVB-N from 7.90 mg N/100 g after pasteurization to 29.24 mg N/100 g at the end of their experiment (21 days). After 12 d refrigerated storage, Caglak et al. (2008) registered TVB-N contents of 36.2–67.3 mg N/100 g in modified-atmosphere packaged shucked mussels.

Regarding pH (Fig. 3.2B), values decreased, sharply in clams kept at 15 °C and 25 °C, from initial values ranging from 6.69–6.71 to minima of 6.35 ± 0.16, 5.97 ± 0.06 and 6.06 ± 0.07 on days 6, 2.5 and 1, respectively at 5, 15 and 25 °C. Then, the pH values of clams stored at 25 °C increased steeply.

Herein pH values observed at the start of the storage trials were higher compared to those found by Erkan (2005) for shucked mussel, 5.96, by Cao et al. (2009) for Pacific oyster, 6.30, or by Manousaridis et al. (2005) for mussels, 6.3, but in line with the results reported in Caglak et al. (2008) also for mussel, 6.69–6.72. The initial reduction in pH values commonly occurs is seafood as a result of acid lactic formation ensuing autolytic processes, eventually from the conversion of glycogen to lactic acid as suggested by Cao et al. (2009); while the later increase in pH results from the production of basic compounds from nitrogen deamination (also reflected in TVB-N levels' increase), eventually due to microorganisms' metabolism (Huss 1995). Coincidently, the minima in pH values matched TVB-N levels reaching the concentrations legislated for fish (25–35 mg N/100 g) (see above) or proposed for bivalves (15–25 mg N/100 g; cf. Tosun et al. (2018)). This might suggest unacceptably low quality for Human consumption at those occasions. However, previous studies (Caglak et al. 2008; Erkan 2005) have been unsuccessful at finding a correlation between the pH and the freshness of bivalves as proposed by Pottinger (1948) for oysters (pH = 6.2–5.9 "good", pH = 5.8 "off", pH = 5.7–5.5 "musty", pH = 5.2 and below "sour or putrid").

3.3.3 *Depurated vs. Non-depurated*

We posited that depuration would have an impact on parameters of biological (mortality rate), physiological/commercial (condition index and percent edibility), physicochemical (pH and TVB-N content), microbiological (TVC, Enterobacteriaceae and psychrotrophic bacteria) and sensory quality of commercial-size clams stored at chill temperatures.

Results show (Fig. 3.3) that clams collected in late-Spring, during the period of peak consumption, subjected to depuration exhibit a similar survival trend to non-depurated specimens when kept refrigerated. The logistic model fitted well the data on proportion alive over storage time (pseudo-$R^2 = 0.979$ for depurated and pseudo-$R^2 = 0.996$ for non-depurated clams). The median time to death, t_{50}, practically overlapped, 13.0 ± 0.12 d vs. 12.1 ± 0.06 d respectively in depurated and non-depurated clams.

When comparing the survival rate of native *V. pullastra* and exotic *R. philippinarum* clams in the Tagus Estuary, Anacleto et al. (2013) found that t_{50} of depurated and non-depurated clams maintained at 4 °C were similar, 5 d for *V. pullastra* and 14 d for *R. philippinarum*. Those authors also experimented with higher storage temperature, 22 °C, to find that t_{50} were much shorter, 3 and 4 d. The median time to death, t_{50}, of 5 d was observed by Marin et al. (2005) that experimented with *T. philippinarum* at 18 °C. In another relatively similar experiment with depurated *T. (Ruditapes) decussatus* carried out at three different storage temperatures by Sadok et al. (2003), authors found that t_{50} ranged from 6.5 d for 5 °C, to 16 d for 10 °C, to 5 d for 20 °C. Bernárdez and Pastoriza (2011) observed increasing mortalities of mussels packed in 21% O_2 atmosphere (~atmospheric air) of up to ca.

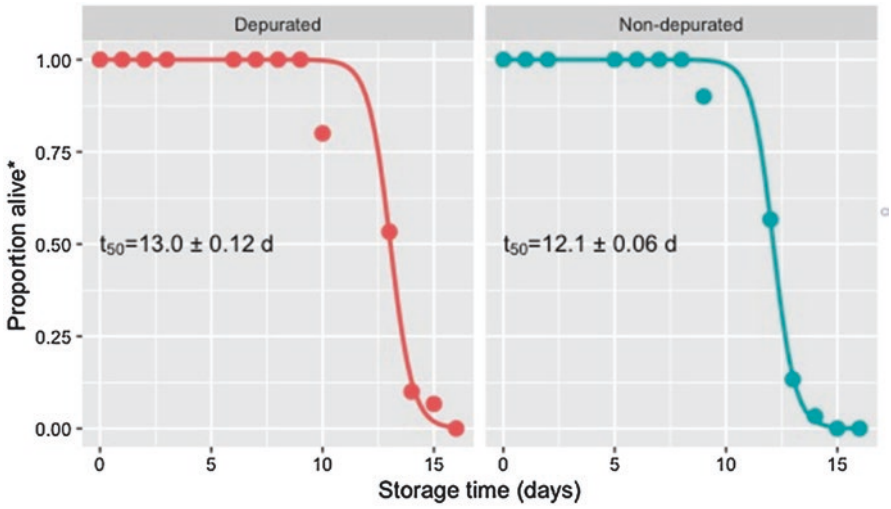

Fig. 3.3 Survival of depurated vs non-depurated clams *R. decussatus* collected in Summer and kept in chill storage at 5 ± 1 °C (n = 50 per treatment)

40% at the end of the 14-d storage period. Our results (t_{50} = 13 d) are close to the t_{50} reported by Anacleto et al. (2013) for 4 °C storage temperature (14 d) and the estimate of 16 days by Sadok et al. (2003) for the 10 °C storage temperature for *R. decussatus* clams. Notwithstanding, herein clams' mortality remained steadily low for up to 9 d, demonstrating clams' resistance to emersion, and corroborating that commercialization of clams in closed net bags outside water is the correct procedure.

In depurated clams, the condition index (CI) decreased and the percentage edibility (PE) increased during storage whereas in non-depurated clams CI remained constant and PE increased, the latter following a similar pattern to that observed for depurated clams (Fig. 3.4). Changes in CI and PE among times of storage were statistically significant (ANOVA, F = 4.69 with p = 0.0013 and F = 6.05 with p = 0.0002, respectively) in the case of depurated clams only. Notwithstanding, the variability of data cautions clear-cut conclusions.

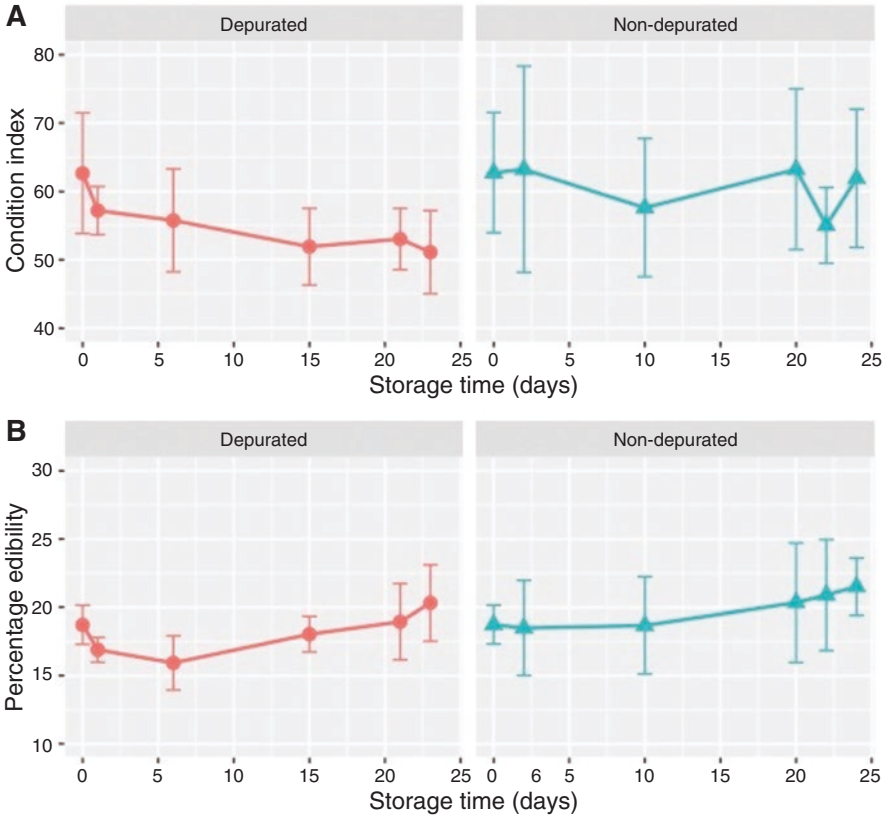

Fig. 3.4 Changes in (**a**) condition index (CI) and (**b**) percentage edibility (PE) of depurated vs. non-depurated clams *R. decussatus* (mean ± SD, n = 60 per treatment)

CI has been used as an ecophysiological measure of bivalve health status (Mubiana et al. 2006) and adopted in international trade as a standard criterion to select the best product (Aníbal et al. 2011). It may be considered as an indicator of fatness and marketability of commercially exploited bivalves (Orban et al. 2004). In this study, the initial CI and PE of clams were relatively lower than those reported by Aníbal et al. (2011) for the species in the Ria Formosa during late-Spring but reflect changing environmental conditions and physiological demands, the latter are assuredly dependent on the seasonality of the species' life/reproductive cycle (Ojea et al. 2004). Anacleto et al. (2013) also found decreasing trends in CI of clams during storage which they attributed to stressful conditions, namely the lack of feed that demands the use of biochemical reserves that decrease body weight (Albentosa et al. 2007). Seemingly, the depuration of clams in this study exacerbated those effects. In contrast, Gonçalves et al. (2009) observed non-significant changes in CI and PE of purified *R. decussatus* clams but in shorter 6-days storage trial (both in air and MAP).

Moreover, TVB-N content of depurated and non-depurated (live) clams stored chilled (+5 °C) exhibited similar exponential patterns (Fig. 3.5A). Exponential models fitted data quite well (pseudo-R^2 = 0.967 in depurated clams and pseudo-R^2 = 0.999 in non-depurated clams). Similar rates of (exponential) increase were found in depurated and non-depurated clams, 0.288 ± 0.058 d^{-1} and 0.256 ± 0.061 d^{-1} respectively. These were slightly lower than the rates estimated previously for post-mortem changes in TVB-N (see above) but higher than the rates reported by Sadok et al. (2003) for *T. decussatus* maintained at 5 °C, 0.104 d^{-1}. In their study, clams' TVB-N content reached ca. 10 mg N/100 after 10 d. Herein, a steep increase in TVB-N levels was observed after days 15–20. Seemingly, clams can sequester ammonia in the hemolymph (Ali and Nakamura 2000) for a few days after emersion, mitigating its toxicity. Afterward, the altered metabolism induced by emersion leads to the production of ammonia and other volatile bases from amines and amino acids as suggested by Sadok et al. (2003). Rey et al. (2012) observed an increase in TVB-N levels of depurated clams (*V. rhomboideus*), oysters (*O. edulis*) and mussels (*M. galloprovincialis*) stored under ozonized slurry ice or flake ice, especially in mussels, during their 6-days storage trial. The TVB-N contents exceeded the 25–35 mg N/100 g thresholds (stipulated for a number of fish species in Regulations (CE) no. 2074/2005 and no. 1022/2008) by day 20–21. If the proposed limits for bivalves (15–25 mg N/100 g; cf. Tosun et al. 2018) are used, then shorter times are estimated 17–18 days.

The pH decreased in the first 1–2 days, markedly in non-depurated clams (from 6.30 to 5.24); then, values increased to pH > 7 (Fig. 3.5B). This increase was more pronounced in non-depurated clams. Again, the initial reduction in pH values commonly occurs is seafood as a result of acid lactic formation ensuing autolytic processes, seemingly more pronounced in non-depurated clams, eventually from the conversion of glycogen to lactic acid as suggested by Cao et al. (2009); while the later increase in pH results from the production of basic compounds from nitrogen (also reflected in TVB-N levels changes). Gonçalves et al. (2009) reported

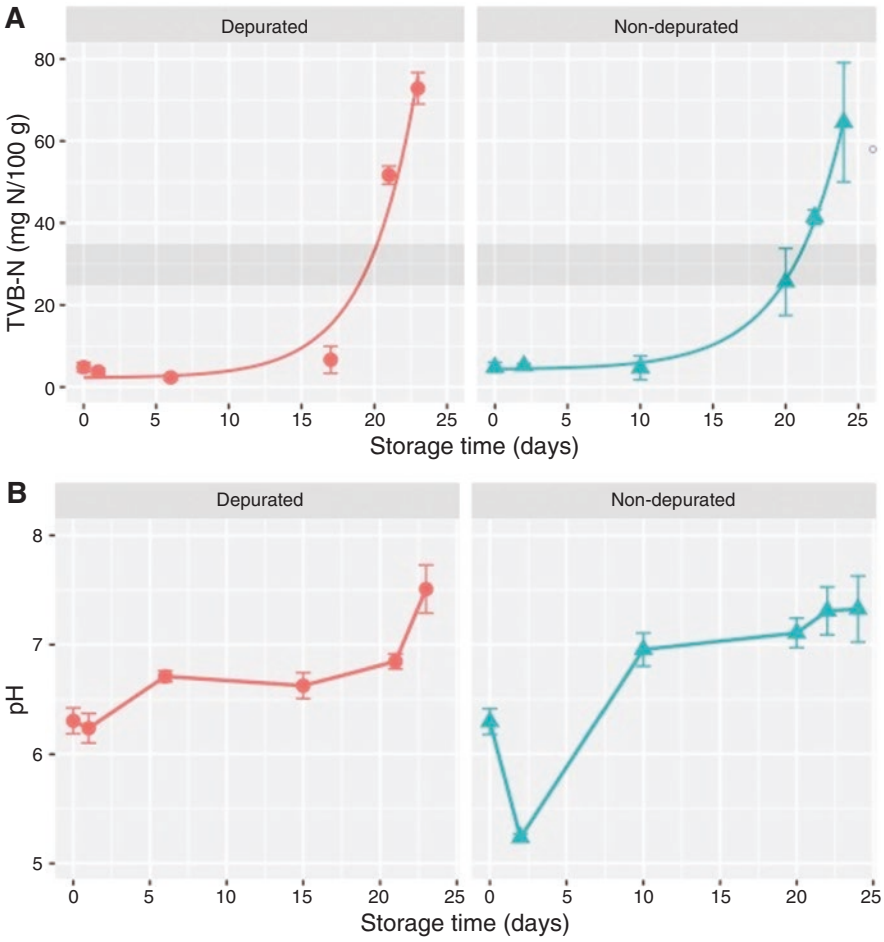

Fig. 3.5 Changes in (**a**) TVB-N content and (**b**) pH in depurated vs. non-depurated clams *R. decussatus* (mean ± SD, n = 180 per treatment)

increasing pH values in purified (commercially depurated) *R. decussatus* clams during a 6-day storage trial, they attributed to low glycogen utilization.

At the start of the trials, TVC averaged 4 log(cfu/g), and then decreased (Fig. 3.6a). This was noticeable and prolonged in depurated clams, to 3.27 log(cfu/g) during the first 6 d; while in non-depurated clams, the reduction was relatively smaller and shorter, to 3.7 log(cfu/g) in <2 d. Afterward, TVC increased in both groups following a similar pattern, but at comparatively higher levels in non-depurated clams (ca. +1–1.5 log(cfu/g)). In the latter, TVC exceeded 5.7 log(cfu/g) just after 20 days of chilled storage and capped at 6.70 log(cfu/g) on day 24, while depurated clams' TVC reached only 4.7 log(cfu/g) on day 23 of the experiment. Regarding Enterobacteriaceae (Fig. 3.6b), abundances remained relatively low, at ca. 1–2 log(cfu/g), during the first 20 days of the trial and then increased steeply in

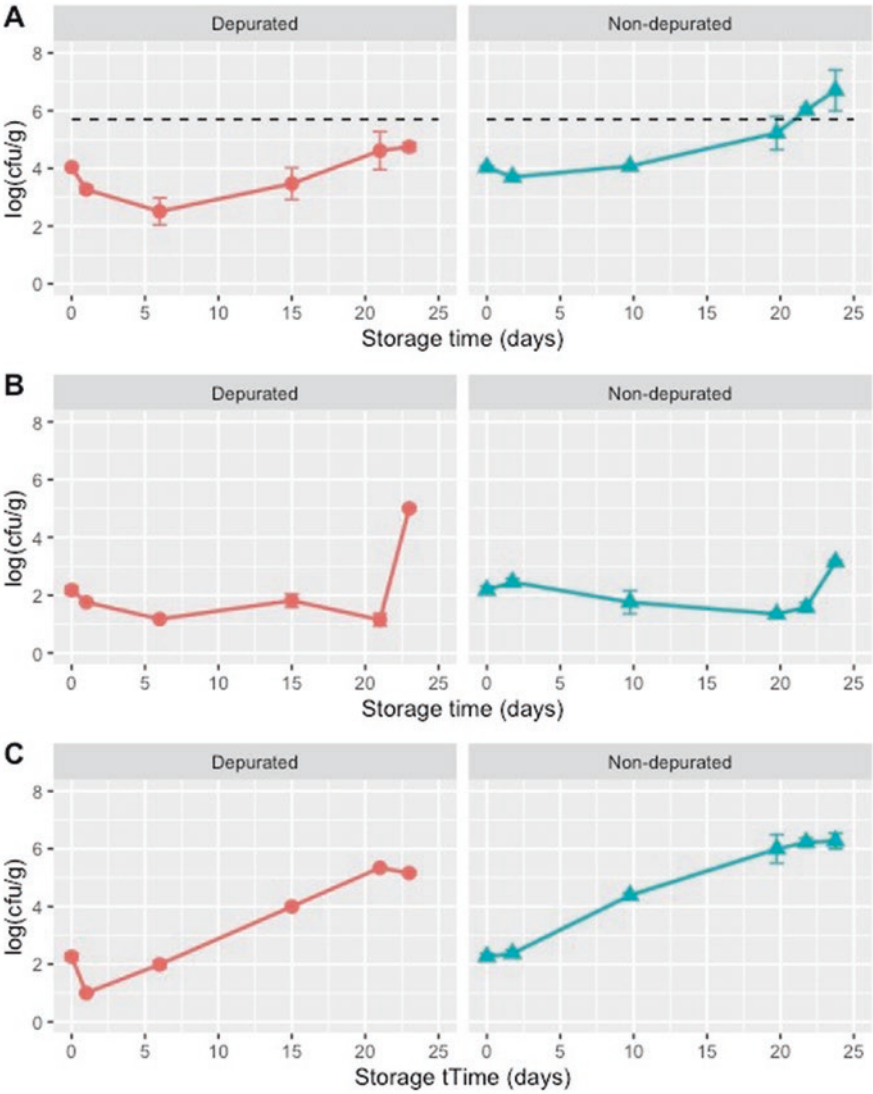

Fig. 3.6 (**a**) Total viable counts (TVC), (**b**) Enterobacteriaceae abundance and (**c**) psichrotrophic bacteria abundance (as log(cfu/g)) in (Summer) depurated vs. non-depurated clams *R. decussatus* (mean ± SD, n = 120 per treatment)

both depurated and non-depurated clams, to 5.0 log(cfu/g) and 3.2 log(cfu/g) respectively. The abundance of psychrotrophic bacteria (Fig. 3.6c) was reduced markedly after depuration, from 2.3 log(cfu/g) to 1.0 log(cfu/g), and then increased linearly to ca. 5 log(cfu/g); whereas in non-depurated clams, their abundance increased steadily from 2.3 log(cfu/g) on day 0 to >6 log(cfu/g) after day 20.

Expectedly, the depuration of clams had a noticeable effect on the microbiota assessed herein. Depuration efficiency is primarily related to size, siphoning activity, and physiological conditions of bivalves (Oliveira et al. 2011). However, other factors, such as the system design, water quality, oxygenation and flow rates, salinity, temperature, shellfish: water proportions, initial level of contamination, type and amount of pollutants, bivalve species and process duration are thought to influence depuration efficiency (Anacleto 2014). Moreover, depuration is highly effective in removing fecal bacterial from bivalves but less or even ineffective to remove other contaminants, e.g. virus, marine vibrios, toxins, or organic chemicals (Lee et al. 2008). El-shenawy (2004) observed reduced levels of microorganisms after the depuration of *R. decussatus* but only after 4 d. Martínez et al. (2009) reported significant reductions in pairwise aerobic plate count (APC) and psychrotrophic bacteria abundances in a set of farmed bivalves after depuration. In clams from the Tagus estuary, TVC were significantly reduced during depuration and later increased during "simulated transport" conditions at 4 °C and 22 °C (Anacleto et al. 2013). Increases of only ca. 1 log(cfu/g) were observed by Rey et al. (2012) in their 6-days storage trial of depurated clams, oysters and mussels using ozonized slurry ice and flake ice. The increase in TVC observed herein exceed the 5.7 log(cfu/g) limit recommended by ICMSF (1986) for bivalves or the 5 log(cfu/g) limit proposed by the National Advisory Committee on Microbiological Criteria for Foods (1992) only in the case of non-depurated clams and after 20 days of storage. Enterobacteriaceae, which include several pathogenic microorganisms, e.g. genus *Escherichia* (incl. *E. coli*), *Salmonella*, *Shigella* and *Yersinia*, may be present in the natural microbiota of foods or can be introduced as a result of post-process contamination (Baylis 2006) and pose food safety concerns since clams are filter feeders (Goulas and Kontominas 2007). Moreover, Enterobacteriaceae are known-causes of food spoilage, since their growth and metabolic activity results in off-flavors and odors and other organoleptic defects arising from the enzymatic breakdown of proteins and lipids (Baylis 2006). Herein, the abundances of Enterobacteriaceae remained low, 1–2 log(cfu/g), within the "borderline limit of acceptability" of 10^2–10^4 recommended in Forsythe (2000), for up to 20 d of storage. Psychrotrophic bacteria are the main contributors to the spoilage of seafood at refrigeration temperatures and thus can be used to estimate the microbial shelf life of seafood (Khan et al. 2005). Herein, only in clams subjected to depuration an initial reduction in the abundance of psychrotrophic bacteria was observed, otherwise, they grew almost linearly during the storage trial (Fig. 3.6c), taking advantage of their ability to grow at low temperatures (Bornert 2000). Considerable high abundances, >6 log(cfu/g), were found in non-depurated clams after 20 days. In contrast, Rey et al. (2012) were able to limit the growth of psychrotrophic bacteria in clams, oysters and mussels during storage for 6 days using ozonized slurry ice and flaked ice.

Concerning microbiological quality, i.e. TVC, Enterobacteriaceae and psychrotrophic bacteria abundances, "unacceptable" levels were reached long after usual consumption period of clams.

In terms of sensory acceptability, depurated and non-depurated raw clams (Fig. 3.7a) exhibited similar median times to rejection, t_{50}, 8.74 ± 0.56 d vs. 7.24 ± 0.56 d respectively. Moreover, on day 10, panelists characterized the clams' fresh, algae odor as very weak (scores of 1.2 vs. 4.2 on day 0) and noticed weak odors of ammonia and sulfides (scores of 1.54 and 1.3 vs. 0) in both depurated and non-depurated clams. In terms of appearance/color, clams were much less bright (scores of 2.8–2.9 vs. 4.3 on day 0) and lost their ivory hue (2.7–2.8 vs. 4.4), becoming milky (2.2–2.3 vs. 1.5) and yellowish (1.5–1.8 vs. 0.5). Similar findings for median time to rejection, t_{50}, were obtained when assessing cooked clams (Fig. 3.7b), 8.72 ± 0.62 d vs. 7.84 ± 0.62 d respectively for depurated and non-depurated clams. Changes in organoleptic attributes were also similar but less pronounced in terms of appearance/color.

In the study of Gonçalves et al. (2009) with live depurated *R. decussatus* clams stored for 6 days, panelists scored specimens at unacceptable levels on day 6, describing specimens' sensory attributes analogously to what was found herein, both in raw and in cooked samples. Moreover, Rey et al. (2012) found Spring-collected, depurated clams, oysters and mussels kept in ozonized slurry ice and flake ice to be acceptable for 4 days in terms of odor, appearance, taste and juiciness. Herein, both depurated and non-depurated clams were considered acceptable for relatively longer periods, plus 3–4 days.

3.4 Conclusion

The survival (t_{50}) of depurated and non-depurated clams stored chilled was similar, ca. 13 vs. 12 days, while their CI and PE changed relatively little (ca. 55–61% and 18–20% respectively). Also, during storage, pH decreased in the first 1–2 days, markedly in non-depurated clams (from ca. 6 to 5) and then gradually increased to values >7 on days 23–25. Concurrently, TVB-N increased exponentially in both depurated and non-depurated clams, exceeding EU limits by day 20. The initial microbial load was fairly low, 2 log cfu/g (Enterobacteriaceae and psychrotrophic bacteria) to 4 log cfu/g (TVC). Expectedly, after depuration microorganisms' abundance decreased, more pronouncedly (1–2 log cfu/g) in TVC and psychrotrophic bacteria. Afterward, abundances grew substantially to 5 log cfu/g at day 24. Enterobacteriaceae abundance remained constant till day 20 and then increased sharply. Similar dynamics of microorganisms' abundance were found for non-depurated clams but at comparatively higher abundances. In terms of sensory quality, the acceptability of depurated and non-depurated clams was similar to $t_{50} \approx$ 7–8 days, in both raw and cooked specimens.

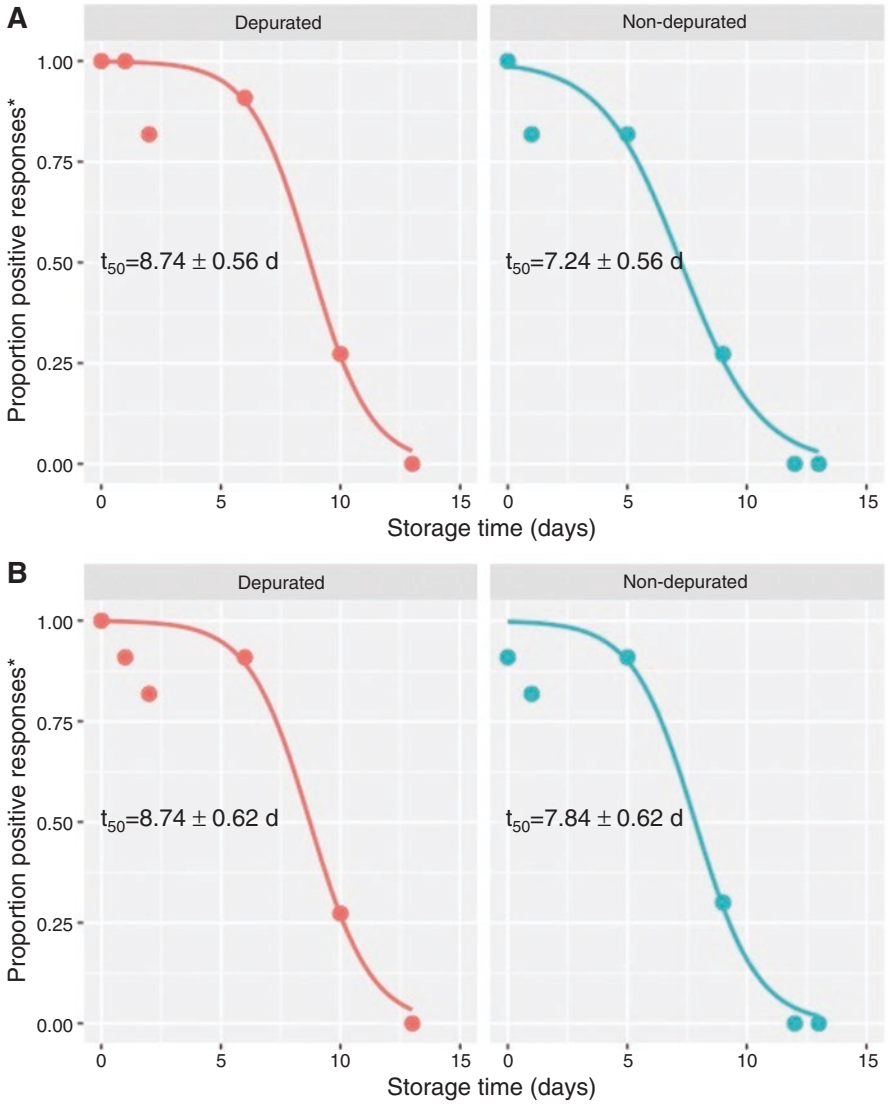

Fig. 3.7 Sensory acceptability of depurated vs. non-depurated (**a**) raw and (**b**) cooked clams *R. decussatus* (mean ± SD, n = 30 per treatment)

In sum, depuration affected in different ways the level but not the general dynamics of the quality parameters assessed during chilled storage of clams. However, eventual safety issues emerge long after habitual storage time and panelists' sensory rejection. Furthermore, our results indicate that the general belief among clam farmers that depuration decreases markedly the condition and quality of the specimens, lowering their marketability and economic revenue is not substantiated, reinforcing the importance of depurating bivalves as a means to ensure public health.

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Part II
Sustainable New Product Development

Chapter 4
Sustainability and Value-Added Products as an Opportunity: Global Acceptability and Sensory Quality of Limpet (*Patella* Spp.) Pâté Enriched with Strawberry-Tree (*Arbutus unedo*) Fruit Extract

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4.1 Introduction

In Portugal, the consumption of seafood products has been increasing around 55.9 kg/capita/year compared to the European average (25.1 kg/capita/year) (PCP 2018). One reason for this trend has been associated with a healthy diet mainly due to nutrients richness such as digestible protein, vitamins, n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA) and minerals (iodine, selenium) (Ribeiro et al. 2019). However, the higher demand for some marine resources leads to limitations of natural stocks in terms of some fish species like sardine, cod and hake (Ramírez et al. 2011). On the other hand, several underutilized fish due to inappropriate color or texture and some fish by-products can be transformed into high-value products (Ramírez et al. 2011). In this context, a need to take advantage of other less exploited marine resources to facilitate the sustainability guarantee and the integrity of the marine environment. In this sense, it is possible to consider a new range of seafood products (transformed/restructured) as an excellent opportunity, enhancing the endogenous products and contributing to obtain higher added value products, enabling other consumption alternatives.

Limpet's (*Patella* spp.) are gastropods mollusk and common species in rocky Atlantic and Mediterranean area and their consumption has been appreciated in several regions of Portugal (Férnandez et al. 2015). However, oxidation is the most important cause of nutritional and sensory quality deterioration of these products.

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Lipid oxidation occurs during handling, processing and storage of fish products leading to loss of quality and acceptability (Estévez et al. 2007).

Natural antioxidants (berries) are employed in food products because of their potential health benefits and safety. There is, therefore, a growing interest in the identification of novel, natural antioxidants for developing functional muscle foods with enhanced nutritional and health properties. The antioxidant activity of plant/fruit extracts is of particular interest both because of their beneficial physiological activity on human cells and their potential to replace synthetic antioxidants, used in foodstuffs (Heinonen 2007).

The new agricultural and reforestation policies have been driven by increasing consumer awareness and demand conducting to changes in several areas such as environmental preservation, maintenance of biodiversity, sustainability in the use of natural resources and social responsibility.

Climate change is increasing temperatures globally and drought in many regions and if this continues, the resilience of many ecosystems will likely be exceeded, altering their structure and function. The Mediterranean region is considered as one of the most fire-prone regions of Europe. In recent years, in Portugal, an alternative strategy has been chosen for low population density and economically depressed territories, using the use of wild resources, (e.g. *Arbutus unedo*) thus increasing its plantation. The main objective of this strategy is to generate economic value and sustainably revitalize the local economy.

4.1.1 Limpets

Limpets of the genus *Patella* (Gastropoda: Patellidae) are grazing gastropods inhabiting rocky intertidal areas of the East Atlantic and the Mediterranean coast in temperate latitudes. They have a key role in controlling algal coverage and, consequently, the ecological succession and biological communities established in intertidal areas (Jenkins et al. 2005; Coleman et al. 2006a, b). As key intertidal grazers, patellid limpets play an important role in macroalgal and biofilm community dynamics, providing valuable information about the food quality and availability (Branch 1985) and nutritional content (Brazão et al. 2003). Due to essential nutrients present in biofilms and/or macroalgae communities, grazer organisms have high nutritional value, being a popular group of edible organisms and one of the most commercially important species in some areas (Silva et al. 2018).

Limpets have been widely exploited for human consumption since the Palaeolithic period (Turrero et al. 2014), in many places around the world such as Mexico and South of the United States (Pombo and Escofet 1996), Azores (Martins et al. 1987; Hawkins et al. 2000), and Chile (Olivares-Paz et al. 2006). Likewise, in Spain, particularly in the Canary Islands (Moro and Herrera 2000) and Asturias, this resource is highly appreciated in gastronomy (Casal et al. 2018).

The growing demand for these high-priced seafood beckons for aquaculture to offset the scale. Aquaculture of limpets, although extremely limited, is currently

being developed with hopes to supply the market with sustainably sourced seafood, improve wild stocks and produce key medicines (Corpuz 1981; Harris and Markl 1999; Hua 2014). Much of the attention for limpet aquaculture is on developmental biology; however, the market demand to expand farming of mollusks has beckoned for limpets to be produced for food. There are many issues in limpet aquaculture because of their sensitive nature and complex environmental and biological requirements, most of which is still unknown in laboratory environments (Mau and Jha 2018). Culture system technology for farming various species of limpets has been sporadically developed and, by default, follows the protocols of mollusk aquaculture in general (Mau and Jha 2018). Many of the limpets used in aquaculture have only been studied for one life stage (i.e. juvenile nutrition trials, broodstock spawning trials) with few species' life cycles closed. The fact that there are missing pieces to the puzzle in culturing a species from larvae to successful adult reproduction results in a deficiency in limpet farming protocols available for commercial-scale operation. The culture system design for limpet aquaculture addresses two major concerns: their ability to firmly attach to the substrate and their sensitive feeding behaviors. When limpets suction to the substrate, moving them from tank to tank poses mortality risks (up to 50%), and their preference for vertical surfaces, slow grazing movements and nocturnal feeding tendency makes feeding them more difficult to culture than the active abalone (Hua 2014). The critical step in successfully developing limpet aquaculture on a commercial level is inducing gametogenesis and spawning animals in captivity. To do this, the focus in limpet research is largely pertaining to inducing final maturation and investigating how to routinely spawn animals in a way that does not result in mortality of the broodstock (Mau and Jha 2018). The improvements of farming techniques for limpets have primarily been focused on spawning and reproduction, predominantly suiting larval development studies as opposed to food production. Many of these developmental biology studies utilize aquaculture and laboratory-controlled spawning events because it is difficult to study in wild limpet larvae. Researchers collected wild, mature animals during the observed spawning season for a given species and kept the animals in flow-through systems until they induced spawning (Kay and Emlet 2002). But spawning healthy limpets naturally in laboratory conditions is shown to be difficult, and alkaline solutions, vigorous aeration, thermal shock, desiccation and artificial insemination are used alternatively—both independently and in combination (Corpuz 1981; Kay and Emlet 2002; Perez et al. 2007; Reynoso-Granados et al. 2007; Aquino De Souza et al. 2009). Spawning of limpets is important, but also difficult and often the limiting procedure for scientists studying developmental biology or farmers intending to produce juvenile animals for market. Spawning events and larval rearing are inherently difficult because they require controlling for environmental and biological parameters during the animals' most dynamic and vulnerable life stage. The most practical method for spawning limpets appears to be injecting gonadotropin-releasing hormone (GnRH) directly into the animal's gonad (Hua and Ako 2012). Once broodstock limpets undergo spawning, the eggs and sperm are collected separately and carefully fertilized with minimal or no aeration in small hatching containers. When the fertilized eggs develop over 12–24 h, the larvae are

collected and rinsed. Larval systems for limpets follow the lead of sea urchin and abalone larval systems. The optimal set-up is a conical tank with moderate, central aeration allowing for good circulation within the tank to promote proper water simulation. These larvae are offered cultured microalgae in their pelagic larval stage, lasting for a few days to a few weeks depending on the species. Once animals are considered competent, they are transferred to a nursery system for settlement and metamorphosis (Mau and Jha 2018). It has been found that limpets prefer vertical surfaces to settle on, so glass or plastic settlement plates designed to the increased vertical surface area are placed in the nursery system (Corpuz 1981; Hua 2014). These settlement plates are also inoculated with microalgae and exposed to a light source to produce a biofilm that serves as the primary food source for developing juvenile limpets. Overall, the movement to formulated diets from natural biofilm diets and routine spawning protocols is the next step in successful commercial aquaculture. Without the sustainable reproduction of broodstock, grow-out juvenile limpets will not be deemed feasible. The development of commercially viable diets of limpets will also remain at bay provided the technology to rear limpets in laboratory settings is not developed.

4.1.2 Strawberry-Tree

The *A. unedo* represents an autochthonous species of the Mediterranean basin, associated with the under-grove of oak stands and holm oak. According to the FF study (Forest Forum) and from an environmental point of view, the exploitation of the *A. unedo* has a strong impact on the landscape by protecting and rehabilitating the soil, since it has an excellent clamping action, improving and protecting soils, reducing the risk of erosion and promoting an increase in soil biodiversity and organic matter. The same study states that the *A. unedo* is adapted to the general soil and climatic conditions of the territory of mainland Portugal since it is a soil indicator species that has not lost its fertility and is resilient to the fire passage and develops a rapid regeneration and recolonization capacity after the occurrence of forest fires. Due to the abundance of its blooming, the *A. unedo* is a species with apiculture interest (Correia and Oliveira 2002) and according to Franco (2013), these characteristics make the *A. unedo* an interesting species for reforestation programs and forest fire prevention measures. In recent years, the cultivation of strawberry-tree has been growing substantially. In the future, the production scale will increase and will be necessary to ensure the marketing and distribution of the fruit.

The Mediterranean forest is a heterogeneous ecosystem composed mainly of Holm oaks (Quercus ilex L. subsp. ballota (desf.) samp.), considered to be the ‘climax vegetation’, and secondary formations of scrublands originated from the degradation of the climax vegetation. Some particular wild fruits from the Mediterranean forest have been used for ages in rural south-western Spain and Portugal as alternative sources of food or in folk medicine. Nowadays, there is an increasing demand for healthier food products amongst consumers, and that includes muscle foods free

of so-called chemical additives. The usage of natural preservatives to extend the shelf life of fish products is a promising technology since many vegetal substances have antioxidant properties (Takyar et al. 2019).

The oxidation of major components of fish (lipids and proteins) is the most important reason for the deterioration of the nutritional and sensory quality of muscle foods. These oxidative reactions cause irrevocable changes in color, taste, flavor and texture, decreasing fish shelf life. Oxidative changes in foods are mainly associated with the presence of free radicals that lead to the production of different compounds (e.g. aldehydes and ketones) responsible for the development of rancid flavors and changes in the color. Besides membrane phospholipids, the complex mechanisms involved in oxidative reactions also affects proteins (Xiong 2000).

The susceptibility of fish products to oxidative processes depends, amongst other factors, on the fatty acid composition of lipids and the level of antioxidants in the environment. Regarding the lipid composition, the most important factor is the degree of unsaturation of the lipid itself (Shahidi and Wanasundara 2002). Most polyunsaturated fatty acids (PUFA) are generally contained in muscle phospholipids, which are consequently more prone to undergo autoxidation than the triacylglycerol fraction.

The demand for natural antioxidants has recently increased because of the toxicity and carcinogenicity associated to synthetic antioxidants (e.g. BHT, propyl gallates, etc.). At present, fruit phenolics (e.g. *A. unedo* and *Rosa canina*) are being applied to various food products claimed to have health-benefits (e.g. functional foods) due to their antioxidant, antimicrobial or therapeutic effects. Amongst the different bioactive substances in wild fruits, phenolic compounds including flavonoids, tannins or phenolic acids have concentrated considerable attention as they have been reported to enhance human health (Heinonen 2007).

The interest of using plant antioxidants in the manufacture of foods goes beyond the antioxidant effect in the food as some of these substances have been proved to display additional health benefits (Heinonen 2007; Apak et al. 2007). Epidemiological studies have shown an inverse correlation between the intake of fresh fruit and vegetables and the risk of cardiovascular diseases and certain forms of cancer (Rice-Evans et al. 1997; Steinmetz and Potter 1996).

The antioxidant activity of phenolic compounds in fruits, vegetables and spices is mainly due to their redox properties and chemical structures, which can act as reducing agents, free radical scavengers, metal chelators and singlet oxygen quenchers (Pizzale et al. 2002; Zheng and Wang 2001; Rice-Evans et al. 1997). Plant phenols may also regenerate other antioxidants and act synergistically with chain-breaking antioxidants under other sets of conditions (Pedrielli and Skibsted 2002).

Nowadays, the increasing demand for convenience foods and the emerging markets for precooked and restructured products call for more options to prevent oxidation and off-flavor development in fish products after cooking (Vuorela et al. 2005).

A wide range of plant phenolics (berries) have been evaluated and tested for their antioxidant properties against lipid and protein oxidation in unsaturated marine oils, meat and fish muscle model systems (Heinonen 2007; Wanasundara and Shahidi

1998; Estévez et al. 2007). Scientists have demonstrated that some fruits and vegetables are a good source of natural antioxidants due to their flavonoids and phenol acids content (Gil et al. 2002; Pantelidis et al. 2007; Wang and Lin 2000).

Extracts of *A. unedo* and *Rosa canina* have shown high antioxidant activities in vitro (Ganhão et al. 2010a), and the addition of these extracts to porcine burger patties and frankfurters has resulted in delaying lipid and protein oxidation (Ganhão et al. 2010b; Vossen et al. 2012; Ganhão et al. 2013).

In conclusion, wild Mediterranean fruits, contain phenolic compounds namely flavonoids and phenolic acids, which compose two large and heterogeneous groups of biologically active non-nutrients. *A. unedo* could serve as an excellent source of high added-value phytochemicals for industrial uses (Ganhão et al. 2010a).

4.1.3 New Food Product Development: limpet's pâté Enriched with Arbutus unedo - Study Case

Limpets are molluscs widely used in food and much appreciated in many regions. The consumption of fishery products rich in polyunsaturated fatty acids has been increasing through the filleted products and restructured products. Food oxidation is the most important cause of nutritional quality deterioration of fish products. Recently, there has been an increase in the utilization of natural antioxidants of vegetable origin in substitution of the synthetic antioxidants, namely in the preparation of restructured animal products such as burgers, sausages and pates. Phenolic compounds from fruits and vegetables (e.g. *A. unedo*) have recognized antioxidant properties and therefore, they are currently considered as good alternatives to synthetic antioxidants in the food industry. In addition to their intense antioxidant activity, there is a general belief that the intake of these substances has health benefits.

In the present study, the effect of *A. unedo* extract (AU), at two concentration levels (3% and 6%; PAU3, PAU6), on sensory stability and global acceptability of limpet's pâté, during 90 days at refrigerated storage, was investigated.

4.1.3.1 Limpets (*Patella* Spp.) Collection and Preparation

The limpets were collected from the beach of Portinho da Areia Norte in Peniche (Portugal) during June 2018. The organisms were removed from the surface rocks by hand with knife help and then were transported in sea water at refrigerated conditions to the laboratory for preparation: washing with salt water, shell removal with knife, sand fragments with the dowel, re-washing with salt water, draining the core for approximately 30 s, weighting, sealing in a vacuum bag and stored at −80 °C until processing.

4.1.3.2 Strawberry Tree (*Arbutus unedo*) Fruits and Extract Preparation

The strawberry tree fruits were harvested in autumn at the stage of full ripeness in the center region of Portugal and transported to the laboratory at appropriate storage conditions (5 °C). On arrival, the fruits were selected, cleaned, sorted to eliminate damaged and shriveled fruits, weighted, sealed in the vacuum bag and frozen at −80 °C, until extract preparation.

For the antioxidant extraction, a modified method described by Ganhão et al. (2010b), was performed.

4.2 Methods

4.2.1 Processing of Limpet's pâté Enriched with Arbutus unedo

The pâté formulation consisted in selected raw materials and ingredients, in specific range concentrations, that resulted from preliminary studies and appropriate processing method (data not shown). The limpet's pâté was manufactured as shown in Fig. 4.1 and according to Estévez et al. (2007), Sánchez-Zapata et al. (2011) and Nielsen and Jacobsen (2013) with some modifications.

In the standard formulation of pâté, the ingredients were as follows (per 100 g of product): 62 g of limpets, 30 g of concentrated mix and 8 g of vegetal oil. Three batches of limpet's pâté was performed according to antioxidant added: synthetic antioxidant, BHT at 0.01% (ID: PCON), *A. unedo* extract at 3% (ID: PAU3) and 6%

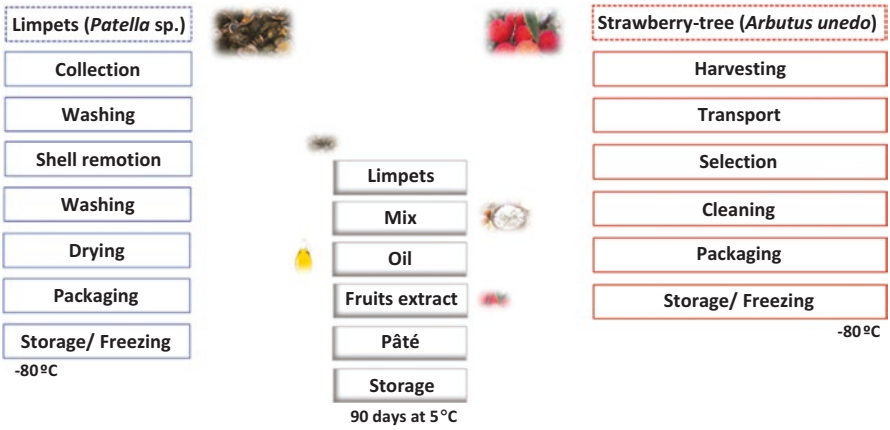

Fig. 4.1 Experimental set-up applied to limpet's pâté processing with incorporation of natural antioxidant additive from strawberry-tree (*A. unedo*) fruits

(ID: PAU6). The synthetic antioxidant, butylated hydroxytoluene (BHT) was added to the pâté formulation according to Commission Regulation (EU) N° 1129 (2011).

The limpet's pâté batches were manufactured as follow: firstly, the limpet's core was steam heat-treated at 100 °C for 10 min in an electric oven (Foinox, MM 100 E Ecomix, France) and chopped in a cutter (Robot coupe, R8 V.V,) until obtaining a limpet's paste homogeneous: first at 1500 rpm for 3 min and second at 2000 rpm for 3 min. After this process, the potato starch mixture and sunflower oil were added until obtaining a homogeneous paste (3000 rpm for 5 min). After, the pâtés were packaged in a glass bottle and subjected to heat treatment at 80 °C for 30 min in a water bath and cooling at room temperature before stored at refrigerated storage temperature (5 °C) for 90 days in the dark.

After processing, the nutritional characterization and total phenolic content were performed in all pâté samples (PCON, PAU3 and PAU6). Also, sensory stability was evaluated during refrigerated storage (5 °C) at 0, 30, 60 and 90 days.

4.2.2 Nutritional Characterization and Total Phenolic Content of Limpet's pâté Enriched with Aqueous Extract of Strawberry-Tree (Arbutus unedo)

The proximate composition of all limpet's pâtés samples (moisture, protein, fat, carbohydrate, ash, fiber) was determined according to the AOAC procedures (AOAC 2000). Total phenolic content (TPC) was determined by Folin-Ciocalteau method reported by Yu et al. (2002) with slight modifications. All analyses were performed in triplicate.

4.2.3 Sensory Analysis of Limpet's pâté Enriched with Aqueous Extract of Strawberry-Tree (Arbutus unedo) During Refrigerated Storage

Sensory analysis was performed by three tests: descriptive analysis, preference and acceptability to evaluate all limpet's pâté samples according to Meilgaard et al. (2007). Twelve trained panelists, age between 20 and 50 years, that know the basic requirements of sensory sensitivity (ISO 8586-1 1993) inadequate conditions (ISO 13299 1995), identified and discriminated the sensory attributes of samples.

The Quantitative Descriptive Analysis (QDA) (Meilgaard et al. 2007) was used to determine the sensory profile. The panelists were asked to mark the perceived intensity of each sensory attribute (sensory profile) like appearance (color and superficial texture), flavor (sea, wild fruits and rancid flavor) and texture (adherence, creaminess and oiliness) from 1 to 5 as observed in Table 4.1.

Table 4.1 Description of each sensory attribute intensity from 1 to 5, like appearance (color and surface texture)

Intensity					
Appearance	1	2	3	4	5
Color	Light – Green (lettuce)				Dark-green (olive fruit)
Surface texture	Homogeneous (gelatin/jelly)				Heterogenous (Rice wafer)
Flavor	1	2	3	4	5
Sea flavor (sea air shellfish)	Imperceptible	Slightly noticeable/ perceptible	Moderately noticeable/ perceptible	Highly noticeable/ perceptible	Extremely noticeable/ perceptible
Wild fruit flavor	Imperceptible	Slightly noticeable/ perceptible	Moderately noticeable/ perceptible	Highly noticeable/ perceptible	Extremely noticeable/ perceptible
Rancid flavor	Imperceptible	Slightly noticeable/ perceptible	Moderately noticeable/ perceptible	Highly noticeable/ perceptible	Extremely noticeable/ perceptible
Texture	1	2	3	4	5
Adhesion	Margarine				Cream of "Serra da Estrela" cheese
Creaminess					
Oilness	Dry (potato puree)				Juiciness cow butter
Residual	1	2	3	4	5
Residual flavor (after 20 s)	Imperceptible	Slightly noticeable/ perceptible	Moderately noticeable/ perceptible	Highly noticeable/ perceptible	Extremely noticeable/ perceptible

The acceptance and the preference test took place shortly after the descriptive test, with the same samples presented. After the panelist evaluates each descriptor and its intensity, he had to evaluate his acceptance of the product in a 9-point hedonic scale according to the method described by (Meilgaard et al. 2007).

4.2.4 Statistical Analysis

For each limpet's pâté, three samples were performed, and all the analysis was carried out in triplicate. The results are expressed as mean values and standard deviation (SD). All data were subjected to analysis of variance (ANOVA) using Statistica™ v.6.1 Software from Stasoft (StatSoft, Inc. 2007). The homogeneity of variance and normality of data has been adequately validated and when these are not found, the nonparametric Kruskal–Wallis test was used (Zar 2010). Statistically significant differences (p-value <0.05) between samples were determined according to Tukey HSD (Honestly Significant Difference).

A principal component analysis (PCA) was performed to evaluate distribution patterns based on the sensory descriptors (appearance: color and surface texture; flavor: sea, wild fruits and rancid; and texture: adherence, creaminess and oiliness; and residual flavor) and the time-point sampling (0, 30, 60 and 90 days of storage). The PCA was used to identify underlying variables (components) that explain the intercorrelations within a set of observed variables (measured data). Therefore, PCA allows associations between variables, reducing the dimension of the data matrix. The principal components provide information on the most meaningful parameters, which describe a whole data set affording data reduction with minimum loss of original information. These are calculated by the linear combination of original variables and they adequately represent the original data (Abdi and Williams 2010; Jollife 2002). The positions of original variables in the principal component plot relevantly represent their interrelations (Abdi and Williams 2010). Thus, if the variables are in the opposite position, then the given variables are negatively correlated. However, if the variables are very closely located, their interrelation is strong and positive. Hence, the graphical representation of the objects investigated in the plot is very useful in detecting their possible association. Moreover, in the principal component biplot, simultaneously representing the objects and the variables, it is possible to detect those variables which are associated with the group formed from closely located objects and in this way the mutual relationships among the objects and variables can be discovered (Abdi and Williams 2010). Canoco for Windows 4.5 software was used to perform the analysis (Ter Braak and Smilauer 1998).

4.3 Results and Discussion

4.3.1 Nutritional Characterization and Total Phenolic Content of Limpet's pâté Enriched with Aqueous Extract of Strawberry-Tree (Arbutus unedo)

The nutritional composition (moisture, protein, fat, carbohydrate, ash, fiber) and total phenolic content of the studied limpet's pâté samples (PCON, PAU3 and PAU6) are shown in Table 4.2.

The results obtained showed that similar energy value was observed on both limpet's pâté samples enriched with 3% (193.70 Kcal.100 g^{-1}) and 6% (195.49 Kcal.100 g^{-1}) of *A. unedo* extract, compared to control pâté (192.19 Kcal.100 g^{-1}) (p-value >0.05). The identical nutritional composition between pâtés samples was due to similar characteristics of ingredients used and type of processing. Écharte et al. (2004) reported a similar nutritional composition of manufactured pâté with different fish species as salmon, anchovy and cod (200–301 Kcal.100 g^{-1}) compared

Table 4.2 Effect of *A. unedo* fruits extract on moisture, nutritional composition and total phenolic content (TPC) of limpet's pâté. Results are presented as mean ± standard deviation of three replicates

Parameters	PCON	PAU3	PAU6
Moisture (%)	65.54 ± 0.15[a]	65.50 ± 0.16[a]	65.23[a] ± 0.07[a]
Fat (%)	12.60 ± 0.23[a]	12.67 ± 0.45[a]	12.88 ± 0.39[a]
Protein (%)	11.89 ± 0.03[a]	11.82 ± 0.03[a]	11.87 ± 0.07[a]
Carbohydrate (%)	7.81 ± 0.40[a]	8.07 ± 0.52[a]	8.04 ± 0.60[a]
Ash (%)	2.16 ± 0.20[a]	1.92 ± 0.20[a]	2.00 ± 0.20[a]
Fiber (%)	0.00 ± 0.00[a]	0.22 ± 0.02[b]	0.80 ± 0.02[c]
Energy value (kcal.100 g^{-1})	192.19 ± 1.30[a]	193.70 ± 2.53[a]	195.49 ± 1.01[a]
TPC (mg GAE.g^{-1})	22.44 ± 1.87[a]	26.50 ± 1.28[a]	30.65 ± 0.36[a]

PCON—limpet's pâté with synthetic antioxidant; PAU3—limpet's pâté manufactured with addition of 3% of *A. unedo* L. fruits extract; PAU6—limpet's pâté manufactured with the addition of 6% of *A. unedo* L. fruits extract.
Different lower letters, in the same line, indicated significant differences at p-value <0.05

to traditional pâté processed with liver pork (249–334 Kcal.100 g^{-1}) (Estévez et al. 2006). The two relevant constituents that intensity contributes to the energy value found are the moisture and fat.

The analysis of the proximate composition of all pâtés revealed no statistically significant differences between samples since presents similar moisture, fat, protein and ash contents (p-value >0.05). However, a significant effect on the crude fiber content of among the pâté samples was observed, from PCON to PAU3 and PAU6 (p-value <0.05). The addition of 6% of fruit's extract into limpet's pâté lead to a significant increase of 0.6% of fiber content compared to extract level of 3% (0.22%). This fact was expected since the strawberry-fruits revealed a high content of fiber (8–18%), as reported by Ruiz-Rodrigues et al. (2011). The evaluation of limpet's pâté samples regarding the benefits of adding *A. unedo* extract as a natural antioxidant additive at two concentration levels was determined by total phenolic content and results are presented in Table 4.1.

Regarding the total phenolic content of all pâté's samples, it was possible to observe that PAU6 reveals the most abundant content (30.6 ± 0.4 mg GAE.g^{-1}) followed by PAU3 sample (26.5 ± 1.3 mg GAE.g^{-1}) and PCON samples (22.4 ± 1.9 mg GAE.g^{-1}) just after the processing. This effect reveals the richness of *A. unedo* in phenolics compounds (data not shown) that agree with studies reported by several authors (Barros et al. 2010; Ganhão et al. 2010b, Rui-Rodrigues et al. 2011). According to Pallauf et al. (2008) *A. unedo* fruits is a good source of antioxidants and can play an important role in human nutrition (Rui-Rodrigues et al. 2011). Moreover, biological effects as antibacterial, anti-inflammatory and anti-carcinogenic of phenolics compounds are related to their free radical scavenging and antioxidant activity (Barros et al. 2010).

4.3.2 *Sensory Analysis of Limpet's pâté Enriched with Aqueous Extract of Strawberry-Tree (Arbutus unedo) During Refrigerated Storage*

The effects of incorporated extracts from *A. unedo* fruits at level 3% and 6% on sensorial attributes of limpet's pâté is presented in Fig. 4.2.

As a dimension-reducing technique, the PCA results led us to two principal components that together accounted for 70.6% of the overall variability of the data

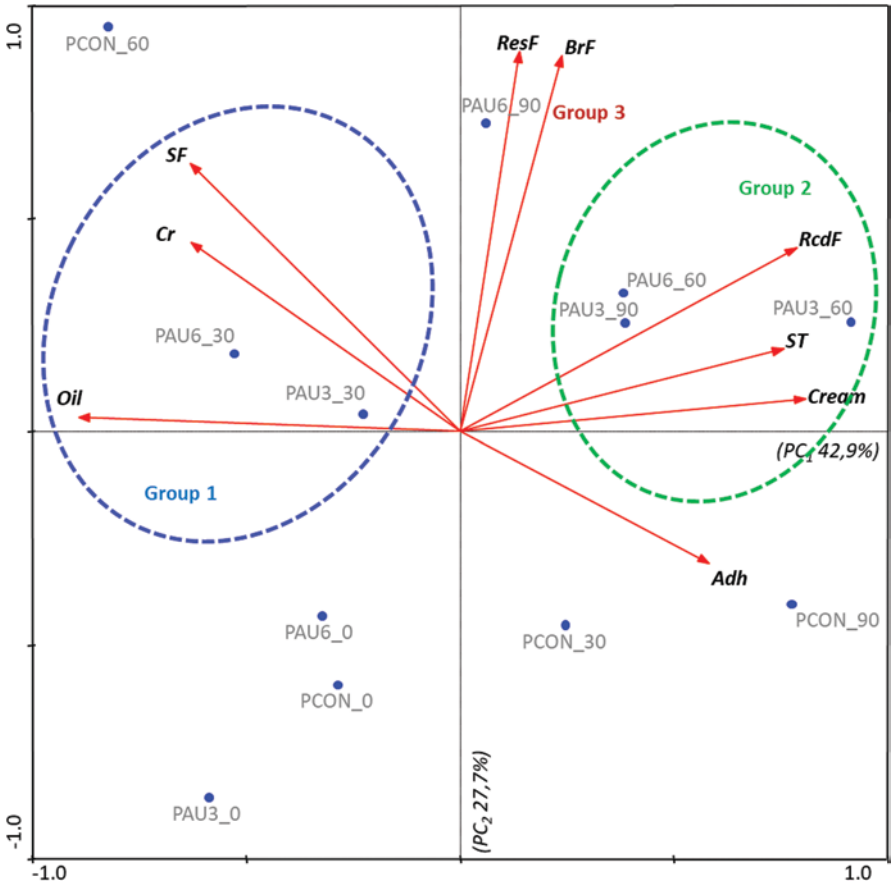

Fig. 4.2 PCA biplot displaying the position of pâté samples as well as of time-points in principal component plane PC1 *vs.* PC2, for storage times under study (0, 30, 60 and 90 days). Time-points were coded as "PCON" for the control sample and "PAU3", "PAU6" for samples with 3% and 6% of *A. unedo* concentration, respectively; the time is coded as "_0", "_30", "_60" and "_90" for each storage time. Sensorial descriptors were coded as "ResF" for residual flavor, "BrF" for wild fruit flavor, "SF" for sea flavor, "RcdF" for rancid flavor, "ST" for surface texture, "Cr" for color, "Adh" for adherence, "Cream" for creaminess and "Oil" for oiliness

(Fig. 4.2). The PCA biplot illustrates the grouping of time-point relative to sensory descriptors, corroborating how the pâté originated from processing and storage conditions may be grouped based on sensory similarities. Variables that presented higher distances from the center of the diagram were the most relevant to explain the total variability of the data and pattern revealed by the results. Therefore, the three main groups were considered. Pâté with 6% of *A. unedo* extract and the 30th storage day (PAU6_30) was strongly characterized by high sea-flavor (SF), color (Cr) and oiliness (Oil) classifications. Of minor importance but equally sharing the same characteristics is the pâté with 3% of *A. unedo* extract (and for the same storage time) (PAU3_30) (group 1, Fig. 4.2). In contrast, these samples obtained lower values in adherence (Adh). For longer shelf-life, i.e. at 60th and 90th storage day, samples with 3% and 6% of *A. unedo* extract (namely, PAU3_60, PAU3_90 and PAU6_60) were characterized by having high scores for residual flavor (ResF), surface texture (ST) and creaminess (Cream) (group 2, Fig. 4.2). Thus, the increase in storage time increases a sensory decrease regarding sea flavor (SF), color (Cr) and oiliness (Oil) (whose evaluations are lower than the average scores for these descriptors). On the other hand, concerning adherence (Adh), the obtained results allowed us to observe that with longer storage time, this descriptor assumes a greater role at the sensory level (as the classification in this respect is higher). Regarding the residual flavor (ResF) and the wild fruit's flavor (BrF), the results were shown to be relevant to characterize the sample with 6% of *A. unedo* extract after 90 days of storage (PAU6_90). Although the importance of characterizing the pâté with 3% and 6% of *A. unedo* extract, at 90 (PAU3_90) and 60 (PAU6_60) days of storage, respectively, was less relevant, the fact is that the evaluation for these two descriptors is still above average for these two samples. Thus, it is possible to point out that the correlation between these two descriptors is strongly associated with the high sensory perception of pâtés with longer shelf life (group 3, Fig. 4.2).

In overall by the sensorial descriptors evaluated, it is noticeable that panelist did not detect a significant difference (p-value >0.05) in terms of appearance (color and

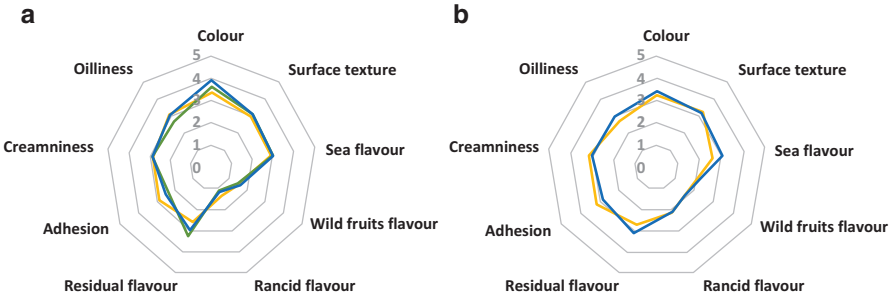

Fig. 4.3 Quantitative descriptive analysis (QDA) of sensorial attributes: appearance (color, surface texture), flavor (sea, wild fruits, rancid), texture (adhesion, creaminess, oiliness) and residual flavor of the limpet's pâtés samples (PCON -, PAU3-, PAU6-) at 0th (A) and 90th (B) storage day at refrigerated temperature

superficial texture) and texture (appearance, creaminess and oiliness) between all samples at the begin and the end of storage (Fig. 4.3).

Our results agree with the findings of Aquerreta et al. (2002), where the four unpleasant sensory attributes (fishy odor, fishy taste, fatness and granularity) of the manufactured pâté with mackerel flesh and tuna liver were evaluated. By sensory evaluation, the trained panelists identified a "strong" and highest intensity especially on odor and taste fishy, in pâté with a higher percentage of Tuna liver (20%) compared to others pâté samples manufactured with low content (10%).

Normally, the seafood products present a fully flavored than compared to freshwater fish due to the accumulation of amino acids that neutralize the seawater salinity. The most remarkable compounds that release the fish flavor and aroma are the sweet amino acid, glycine and savory amino acid, glutamate (Gibson and Newsham 2018). Furthermore, Al-Kahtani et al. (1996) stated that products of lipid oxidation are responsible for the oxidized flavor (also called rancid flavor) that develops in food products during storage. Comparing the results of the rancid-flavor descriptor perceived by panelists, it was evident that pâtés samples with 3% and 6% showed similar behavior regarding this descriptor and PAU6 showed the highest stability concerning to oxidative changes.

The obtained results of flavor-related descriptors denoted no significant difference ($P > 0.05$) with the exception in sea flavor. On the other hand, from the 60th-day storage, descriptors like rancid flavor, wild fruits flavor and residual flavor, revealed statistical differences in all pâté samples (p-value <0.05). Likewise, when storage time increase, a wild fruits flavor stimulus also increases especially in PAU3 and PAU6 pâté samples.

No significant differences were found on the sensorial preference of pâté samples during storage (data not shown) (p-value >0.05). However, and according to the outcomes, the PAU6 pâté samples were evaluated with "most preferred" in all analysis days, followed by PAU3 as "intermediate preference" and PCON as "less preferred" proving the PAU6 sample was well accepted and distinct from the other samples. Interestingly, in respect of sensorial acceptability, the panelists identified as "like moderately" and "like strongly" all samples at day 0 (Fig. 4.4). Throughout storage, the PAU6 pâté samples stand out from the other samples for higher acceptability expressed as "like strongly" and "like extremely".

4.4 Conclusions

The *Arbutus unedo* fruits harvested in the center region of Portugal, revealed a great nutritionally content in carbohydrate and protein with a high content of phenolic compounds leading to lipid stability of limpet's pâté enriched with both concentrations of fruit extract. Both pâtés enriched with 3% and 6% of *A. unedo* extract, have been shown to have sensory stability over the refrigerated storage time. On the other

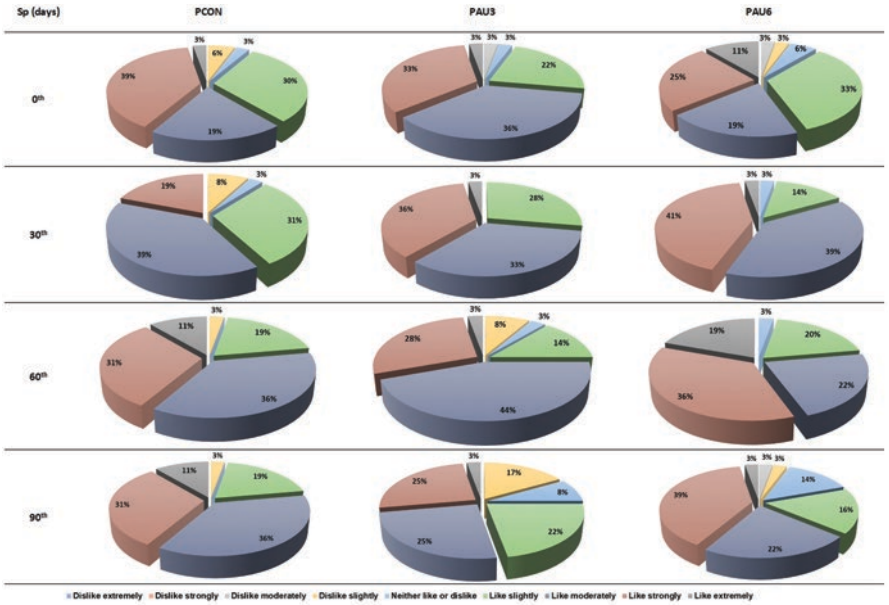

Fig. 4.4 Sensorial acceptability of limpet's pâté (PCON, PAU3, PAU6) during 90 days at refrigerated storage Finally, at the end of storage, a higher differentiation between all pâté samples was observed where the PCON sample was the less preferred. Thus, as referred in the preference test, there is a higher tendency for selected the PAU6 pâté sample as a natural and good alternative of limpet pâté compared with PCON sample

hand, the intensity at sea flavor is losing influence during storage time, however, being a noticeable sensory descriptor for the product that is consumed after 30 days. Additionally, both surface texture and creaminess are the strongest descriptors for longer storage periods. Finally, it is noteworthy that the residual flavor, as well as wild fruit flavor, are more accentuated characteristics for products with longer storage time. Affective sensory evaluations (preference and acceptability) showed a clear tendency of panelists to choose this product.

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Chapter 5
Development of Gastronomic Strategies for the Application and Valorization of New Inverse Emulsions of Vegetable Origin

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5.1 Introduction

According to the 2018 EU Food and Drink Industry Annual Report, the agri-food and beverage industry is the main sector of activity in Europe, with an annual turnover of 1.109 billion euros of exports, 4.57 million workers, more than 294,000 companies and 500 million consumers worldwide. Still in the report referred to, it is pointed out that 13.8% of the European family budget is used in food and beverages, as Portugal represents 18% (FooddrinkEurope 2018).

In the last few decades, there have been large changes in the agri-food industry that have led to growing innovation in food production and supply. The new consumers demand is mirrored in the design and availability of tailor-made food production and idealization, whether from an adequate nutritional, health and wellbeing point of view or from the perspective of convenience, reliability and quality, including sustainable management and ethics of the resources used in its production (PlantFoods 2018).

These challenges are an opportunity for the development of new products and the creation of new niche markets. Therefore, there is R&D investment and particularly in the food technology, biotechnology, and nutrition fields (EMF 2018).

Currently, the use of surplus production and agri-food by-products is very desirable and necessary concerning production and consumption sustainability. Nevertheless, to satisfy the consumer, food innovation strategies must incorporate

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in their structure process not only consumer trends but also direct interaction with the consumer, anticipating their acceptance and commercial potential.

The key to the product-market experience success lies in the balance between responsiveness and proactivity and the short-term success of incremental innovation and the long-term success of more radical innovations. In the globalization phenomenon of the food market, innovation is an essential strategic tool to obtain a competitive advantage, stand out from the competition and meet consumer expectations. However, the more a food product is distinct, the more choices product development teams must make, and these choices must be according to consumer desires. Thus, the successful development of innovative food products requires an excellent understanding of consumers' perceptions, expectations and attitudes towards food products. For this reason, the integration of the consumer in the innovation and development of food product activities can increase the amount of diversified and customer-based knowledge, which helps to reduce the rate of innovation failures. Besides, consumer integration also helps to create innovative ideas and feedback on new product concepts or prototypes (Traynor 2013).

Spreadable creams are essentially water-in-oil emulsions. The lipid phase is usually a mixture of vegetable oils and/or oils and fats of animal origin containing natural dyes (β-carotene), stabilizers, emulsifiers, flavorings, antioxidants, lecithin, and fat-soluble vitamins. The aqueous phase contains proteins, skim milk, where small amounts of other ingredients, such as salt, preservatives, thickeners, and water-soluble vitamins (Nylander et al. 2008).

The emulsions used in this study are of two kinds (Lima 2014):

- oil in water emulsion: for this kind of emulsion the emulsifier must be soluble in the aqueous phase. If anionic or cationic emulsifiers are used, the lipophilic end of the molecule will be adsorbed on the surface of the lipid phase and the hydrophilic end will be at the interface. The charge developed on the surface of the oil droplets will cause the charged particles to repel similarly, and this will prevent coalescence, giving stability to the system. When nonionic emulsifiers are used, stabilization is due to hydration and hydrogen bonding of the hydrophilic end of the emulsifier molecule. A surfactant forms a protective film around the oil droplet, not being very soluble in water, otherwise, it migrates to the aqueous phase and forms a new micelle and, if that happens, oil droplets without protective charges coalesce and the MS breaks down.
- water in oil emulsion: in these emulsions, the hydrophilic part of the emulsifier molecule is dissolved in the dispersed water droplets and the hydrophobic end is oriented towards the lipid phase. In the absence of fillers, the viscosity developed by the orientation of the carbon chains in the continuous lipid phase is probably a stabilizing factor. To form this kind of emulsion, the emulsifier must preferably be soluble in the lipid phase, reduce the interfacial tension between the two phases, form a rigid and non-deformable interfacial film to prevent coalescence, and be quickly adsorbed at the oil interface.

Like traditional butter, spreads have several applications: bread, toast, crackers and other bases, can be used as an appetizer or side dish and/or to prepare other

types of food, including cold meat, roast beef and grilled meat or fish. It is not recommended for people who are allergic to any of its ingredients (Lima 2017).

Mustards are emulsified, oil-in-water (o/w) type vinegar products, in which the continuous phase is water and the dispersed phase, oil. The lipid phase is an oil of vegetable origin. The milled mustard seeds release surfactant phospholipids which help to stabilize the emulsion formed. Mustard creams are used as a seasoning and/or side dish of salads, fried and grilled meats, sandwiches or in the confection of sauces. Mustards are used to add flavor to several dishes and to enhance the piquancy and texture of several types of sauces. They also are important ingredients of the English mustard, the Dijon mustard, vinaigrettes and the Chinese hot mustard (Hrideek 2004).

In the case of these categories of products, the success in the development of new food products by total or partial substitution of certain lipids by plant species and their by-products (e.g. polymers) is based on ensuring the maintenance or improving the sensory characteristics of traditional products; as well as achieving the stability of the product over a period considered enough. New possibilities may rely on providing new sensorial experiences and new applications for these products (Ribeiro et al. 2015).

Many factors contribute to the acceptability of newly launched products both in the market and in the foodservice industry, like the organoleptic attractiveness of the product and its convenience of use. Sensory evaluation is therefore an important step in the food development process as it is its potential gastronomic use (Yang and Lee 2019).

Sensory characteristics, such as appearance, odor, flavor, and texture are included within the important attributes that contribute for the perceived quality of food products (IFT 1981). The food specialist's sensory acceptance assessment may contribute to the understanding of the potential of the food developed and, in the context of consumers' test, to the prediction of the overall success of the product.

The affective test, was the test used in this investigation, using acceptance tests by the hedonic scale and attitude or intention scale tests. With the affective test hedonic scale, the individual expresses the degree of liking or disliking a given product, globally, or concerning a specific attribute. The most used threshold are those of 7 and 9 points, which contain the defined terms located, for example, between "I liked it a lot" and "I disliked it a lot" containing an intermediate point with the term "I didn't like it; I didn't even dislike". The threshold must have a balanced number of categories for taste and disgust. Samples encoded with three-digit and random numbers are presented to the taster to assess how much he likes or dislikes each one, using the previously defined threshold. In the attitude or intention threshold of the affective test, the individual expresses his desire to consume, acquire or buy, a product that is offered him through the attitude or intention threshold. The most used threshold is the verbal threshold of 5–7 points. Coded and random samples can be presented sequentially to the tester to be evaluated using the pre-defined threshold. Defined terms can be, for example, between "probably would buy" to "probably would not buy" and, in the middle "maybe buy, maybe not buy".

The threshold must have a balanced number of categories between the intermediate point and the extremes (Instituto Adolfo Lutz 2008).

Bearing in mind the quite different and innovative flavors that the consumer is used to, concerning simple margarine, it will be imperative, to the product's success, to find flavor combinations that fit its purpose. There is the possibility of looking differently at the combinations of flavors within a dish when using food pairing tools. Food pairing is a scientific method for identifying which foods and drinks are well suited to each other. To understand why the ingredients, combine, it is important to know how humans perceive flavor (Page 2008).

It has long been known that our food experience is extraordinarily complex and involves all our five senses. Although vision—the impact of food color or the presentation—and hearing—the expectation of crunchiness—affects our perception, there is no doubt that our taste experience is composed, for the most part, of the essential taste, touch and smell sensations. The taste sensation is easily correlated with our taste experience. When tasting food, we detect the five basic flavors in our mouth and on our tongue: sweet, salty, bitter, sour and umami. The feeling we experience while biting and chewing food, makes us experience texture, freshness, and pungency. However, on average, only 20% of our taste experience is due to taste and touch. Much more relevant is our smell sense. Through this sense, we can differentiate up to 10,000 different odors. Odors are also known as smells, aromas or fragrances and consist of one or more aroma molecules. The aromas are volatile and reach our smell sense through the air we inhale. We perceive aromas through both the nose (nasal) and the mouth (retronasal) (Kort et al. 2010).

Combining this tool and available knowledge, comes the work of the chef or bartender or another food specialist who shows his experience and ability to generate recipes, techniques, and confections, knowing in advance that the chosen ingredients combine well with each other. To be able to use this application, it is necessary to access the website https://www.foodpairing.com/en/home and the tools available are more than 1500 ingredients, more than 250 drinks, the best combinations, the seasonality filter, unique ingredients, mixology ingredients, aroma filter and aroma wheel. Whenever an ingredient is selected, the algorithm calculates and presents possible aromatic combinations. The greater the aromatic combination, the greater the chance that these ingredients will combine well in a recipe. The tool finds complementary pairs, while the researcher, as a chef, has the task of creating the perfect balance between flavor and texture and to add depth and dimension to the delicacy (Foodpairing 2017).

Finding the right balance seems simple in theory, but it can be the most difficult part of the job when you're in a culinary workshop. It is not possible to build a recipe based only on complementary aromas, which can become uninteresting. As mentioned, only about 20% of our food and drink experience is due to taste and texture, while 80% is due to aroma. However, these factors contribute together to the overall experience and satisfaction and must be taken into account, together and not separately, when building a recipe. Therefore, it is not enough to pair ingredients only with similar aromatic profiles, but also ingredients that have contrasting tastes and textures (This 2006). For example, using bitter to contrast with salty: adding a

pinch of salt when baking a chocolate cake, contrasts with the bitterness of dark chocolate. You can reduce the intensity of a dessert by counterbalancing it with something acidic. Most of the delicacies that arouse our interest are those that exhibit a variety of textures, instead of those that lack texture, such as baby food that becomes boring after a few spoons. Some textures are part of two distinct groups: the soft textures and the crunchy textures. When building a recipe, at least one contrasting texture must be included for each of these groups to give dimension to the dish. This ability is usually innate, because naturally we already have an affinity to create these combinations (soft and crunchy foods), as are the example of french fries with mayonnaise or ketchup or chocolate mousse served with a biscuit or crumble. A mousse (soft texture) becomes more interesting when you add something crunchy like a cookie (Foodpairing 2017).

The aroma profile of culinary ingredients is the starting point of the Foodpairing® computer application and this work. First, Foodpairing® determines the aroma profile of a specific ingredient—with simple gas chromatography coupled with mass spectrometry (GC-MS). From these results, the scientists responsible for the Foodpairing® application extract the aroma data relevant to the human smell sense. For example, a strawberry contains a few dozen different aromas. However, only a few aromas stand out clearly and determine that precise strawberry smell. An aroma has to reach a certain threshold in a specific ingredient to be sensitive to humans. Through potential interactions, some scents that are below this threshold generate a detectable smell. Second, the Foodpairing® application uses scientific techniques, such as data analysis and computational machine learning, to create algorithms that calculate how food and drinks combine. This way, when different foods share certain key aromas, they are more likely to combine well in a recipe. The tool allows the discovery of pairings with considerable ingredients. From dairy to meat, from vegetables to spices, from spirits to coffees, from plants to insects, the Foodpairing® application has traveled the world in search of local products and unknown ingredients (Traynor 2013).

The ingredients combine when they have key aromatic compounds in common. Flavors are important, as our smell is responsible for 80% of our taste experience. These are the sum of the characteristics of any material placed in the mouth, perceived mainly by taste, smell and by the nociceptors and tactile receptors of the mouth (Burdock 1994).

There is a lot of literature regarding the listing of aromatic compounds present in foods. There are databases with ingredients that were analyzed in the laboratory to trace their aromatic profile (Burdock 1994; This 2006; Page 2008; Segnit 2010). Each result when compared to thousands of other profiles determines the best combinations.

According to Heston Blumenthal, in his book The Fat Duck Cookbook (2009), the consumption of food and drinks is part of the most satisfying and multisensory life experiences. Pleasure comes, not only from the oral sensations of taste and smell but also from the sight, feeling and sound that is perceived when eating.

For most chefs, innovation is not seen as a competitive tool from an economic point of view but as a differentiating marketing strategy in which, in most cases, it

is financed by them and where the barriers mentioned are the difficulty in obtaining capital, customer responsiveness, lack of innovative culture in general and lack of qualified human resources. The study supports Gomez et al. (2003) when he mentions the importance of the chef's leadership in the process, adding that his search for innovation is a differentiating factor regarding the competition. Innovative activities improve not only the dish but also the culinary process, efficiency, flexibility, productivity and compliance with hygiene and food safety standards by formalizing the routines resulting from the process. Innovative chefs develop and manage their brand as a fundamental part of the marketing activity as described by Gomez and Bouty (2009). The customer's opinion hardly influences these processes described above, the chef's source of inspiration being his team, his competition and gastronomic fairs and congresses, an idea corroborated by literature (Harrington 2004; Svejenova et al. 2007).

Globally, it can be considered that innovation, in the context of food production, including catering, should be considered a strategy, as there is the possibility of being a differentiating tool for the layout of a brand and in the focus of a market segment; it must be managed in such a way as to produce successful results in which the process's formal organization can lead to accurate reproduction and ensure quality. Cooperation with scientists and external researchers to the production context enhances innovation and the formal management of the process. Innovation requires time and space as creativity seems to emerge more easily outside the pressure of the work context; in the particular case of catering (culinary arts), training and the study of culinary techniques must be encouraged because innovation depends on this factor and not on intuition.

The objectives of this study were to assess the gastronomic potential and possible uses of water-in-oil (60–65% lipid phase) innovative inverse emulsion prototypes previously developed (Lima et al. 2017; Laranjeira et al. 2018), using both sensory evaluation and the Foodpairing® tool and also to develop new gastronomic applications, determining consumer's acceptance.

5.2 Materials and Methods

5.2.1 Samples

Five samples were analyzed—three emulsions of strawberry and bell pepper (one red and one yellow) processed differently, with aqueous vegetable phase and two mustards with red fruits or beet. These products were recently prototyped and are characterized for the additions of vegetables and/or fruit syrups, with no tradition of manufacture or consumption in Portugal, preserving expensive/seasonal raw materials and value surplus/regional by-products and for having nutritional quality. These emulsions have a vegan or lactovegetarian profile, which can be used as substitutes for butter (fat phases using cocoa butter or coconut oil). Traditional

mustards (in vinegar) are distinguished by ingredients, flavors and unusual colors (Lima et al. 2017; Laranjeira et al. 2018).

5.2.2 Gastronomic Potential Evaluation

5.2.2.1 Sensory Evaluation

To carry out a first sensorial characterization of each of the samples and to determine the perception of the respective potential of gastronomic use, a hedonic test was performed using a taste panel (experts—six Chefs and three food professionals), previously established. The general attributes were considered from the descriptors previously generated by the researchers. The individual parameters selected were as follows:- visual appearance and color (on appearance); smell/odor, flavor/aroma, taste persistence (gustatory smell): used a 9-point scale with defined terms situated between "poor" and "excellent";- texture, ointment and acidity (for mustard fruity creams): used a 9-point scale with defined terms situated between "extremely unpleasant"and "extremely pleasant". An overall assessment item (using a 9-point scale with terms defined between "poor" and "excellent") was also presented. In the scope of the test were also measured:- consumption potential and purchase intention: a 5-point scale was used with definite terms between "definitely no" and "certainly yes");- the culinary potential of the samples per se and as a basis for other preparations: a 5-point scale with definite terms placed between "definitely without application" and "certainly with application"); It was also asked to identify the emulsion fat in the case of the first three samples and comments on the potential culinary applications of all creams.

The samples were coded using a three-digit code and analyzed in the laboratory at a temperature of about 20 °C, similar to the one that is customary to use with natural fluorescent lighting.

5.2.2.2 Foodpairing Assessment and Recipe Development

The online Foodpairing® tool was used. Through the data obtained in the sensorial analysis performed on the samples, the main aroma of each sample was identified. This identified ingredient was selected in the online application. Then the other ingredient (s) that composed the emulsions were selected. In view of future developments, it was previously established the context of use of a possible delicacy: for Food Service or domestic consumption, since the level of difficulty of producing the recipe, and the type of ingredients used would have to be different. Then it was considered the order to appear in a possible menu—sauce, canapé, a cold starter, hot starter, main course of fish, meat main course, garnish and dessert.

Following, one or more ingredients were selected from the presented results, calculated by the application algorithm, as being the "best aromatic combination".

A recipe set was developed based on previous results with the online Foodpairing® tool and also based on the culinary know-how creative/aesthetic talent of the researcher. The following aspects were taken into consideration: presentation of the delicacy on the plate; Development of cooking methods in a cooking laboratory environment; presentation suggestion developments. There was a non-systematic evaluation by four members of the research team.

5.2.2.3 Consumer Testing

A script of the tasting menu was established, selecting ten recipes according to their logical sequence in the menu, respecting the place of delicacies: cover, starters, vegetarian dish, a fish dish, meat dish and dessert. Additionally, a sensorial questionnaire in the form of a test book was developed, including the parameters of acceptance; purchase intention; marketing and use potential for each emulsion sample and each delicacy, in agreement with the scales identified in Sect. 5.2.2.1.

Finally, an acceptance test was carried out in a tasting lunch in a pedagogical restaurant for 40 consumers (domestic/food professionals).

5.2.3 Statistical Analysis

Data were treated using the Statistical Package for Social Sciences (SPSS), IMB software version 24.0 and Excel spreadsheet software, Microsoft Office 365, version 16.0.The results were presented in mean ± standard deviation (SD) and frequency. Emulsions were accepted when they obtained an average ≥ 5.0 (equivalent to the hedonic term "neither good nor bad").

5.3 Results and Discussions

5.3.1 Gastronomic Potential Revealed

The panel positively evaluated all emulsions (global appreciation mean values between 5.6 and 7) but none was pointed out as having potential gastronomic use by itself, but always as an ingredient of some composition. These tasters preferred the yellow pepper spread on all aspects except for the smell/odor; in this parameter the preferred one was the red pepper cream (Table 5.1).

The least appealing in terms of the visual appearance, color and greasiness was the strawberry cream because everyone noticed a lack of red color, characteristic of the strawberry and a weak greasiness, due to its high viscosity at room temperature.

Table 5.1 Mean values of the parameters analyzed in the study by the expert's panel, using a 9-point scale with defined terms situated between "poor" and "excellent"

	Strawberry	Yellow pepper	Red pepper	Beet and raspberry mustard	Raspberry and blueberry mustard
Appearance	4,78 ± 2,11	7,67 ± 1,12	6,44 ± 1,51	5,67 ± 1,50	6,78 ± 1,30
Color	4,33 ± 2,00	7,78 ± 0,83	7,00 ± 1,41	5,33 ± 1,58	6,67 ± 1,50
Aroma	6,11 ± 1,96	6,22 ± 0,97	6,67 ± 1,22	6,22 ± 1,39	6,22 ± 1,99
Flavor	6,22 ± 1,79	6,78 ± 1,20	6,44 ± 1,01	5,33 ± 1,73	6,33 ± 1,41
Overall quality	5,67 ± 1,5	7,00 ± 1,00	6,22 ± 0,97	5,78 ± 1,64	6,44 ± 0,88

The second less appreciated was the beetroot and raspberry mustard cream, again by the visual aspect and the color little appealing and also because it is not homogeneous, being the presence of the seeds a depreciative factor, not only in this sample, but like in the others which also had this aspect (strawberry emulsion and raspberry and blueberry mustard) (Table 5.1).

Fats used in the emulsions were always identified (data not shown) (cocoa butter or coconut oil) and led to satisfactory aroma and flavor acceptance levels (>6). Studies related to cocoa butter have shown that it can be used to alter the introduction of fats into margarine and chocolates, making them healthier, to reduce the epidemic problem of global obesity by manipulating the percentages in their emulsions (Norton and Fryer 2012).

The sensory results obtained in this phase of the study may be seen as an important contribution to the future commercialization of the products, since it gives us a perspective of the potential consumer acceptance (Mohamed and Shalaby 2016). A number of studies have been carried out during the development of the products in order to achieve an optimum formulation from the consumer's point of view. Several researches on similar products of the studied ones, confirm the importance of conducting acceptance tests during the development process, even leading to recommendations to reformulations or improvements of the sensorial characteristics of products to be better accepted by the consumer (Nwosu et al. 2014). Other studies reveal that appearance and color are aspects that influence the appreciation of products, which is in accordance to our findings. In comparison with products of similar aromatic profile, the consumer prefers what he is already used to as the reliable product (Racolta et al. 2014).

In our opinion, the study also benefits from using individuals experienced in tasting food, as they more easily were able to identify and name the flavors in the new products.

In relation to the possible gastronomic use of the emulsions (data not shown), none was pointed out as having great potential on its own, to be used alone, but always as an ingredient of some composition. Strawberry sour cream has been suggested only for desserts or sweet compositions; the yellow pepper spread was singled out as a flavoring potential for a white rice or a cooked dough, to finish off risotto or curry such as roasted meat seasoning or sauces ingredient; the red pepper cream was the least appreciated in terms of culinary potential because it was

compared to the mass of pepper and its consequent use; beetroot and raspberry and raspberry and blueberry mustard creams were used as ingredients for vinaigrettes or for spreading on roasted meats, the latter being the second most appreciated in this sensory evaluation.

Addressing the consumption potential (Fig. 5.1), on average the yellow pepper was the sample that showed the best results. The red pepper emulsion and the Raspberry-blueberry mustard showed intermediate results, followed by the strawberry emulsion and with the worst result, the beetroot mustard cream, however, all the samples revealed, on average, a positive consumption potential.

In what regards to the relation to the intention to buy, on average (Fig. 5.2) the yellow bell pepper was the sample that showed the best results. The red bell pepper emulsion and beetroot and raspberry mustard cream showed intermediate results, the strawberry emulsion and the beetroot mustard cream having the worst results, however, all the samples revealed, on average, a positive purchase intention.

5.3.2 Foodpairing Possibilities

There were 33 combinations of ingredients with the Foodpairing® tool for the five prototypes considering possible meal courses which, cross-checked with the tasters' panel, led to 34 gastronomic compositions further developed in a culinary workshop (data not showed).

The chefs' opinion was not always coincident with the ingredients proposed by the application of Foodpairing®. We are aware that the suitability of the ingredients

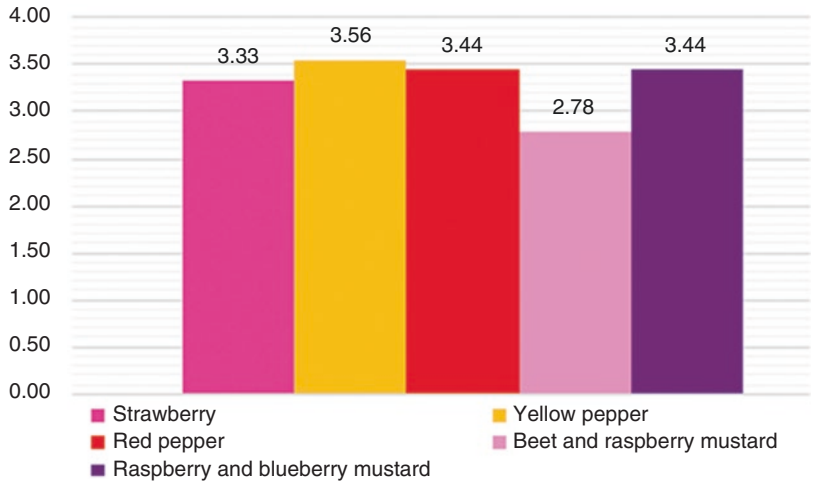

Fig. 5.1 Consumption potential of the samples analyzed by the experts' panel, using a 5-point scale with definite terms placed between "definitely without application" and "certainly with application"

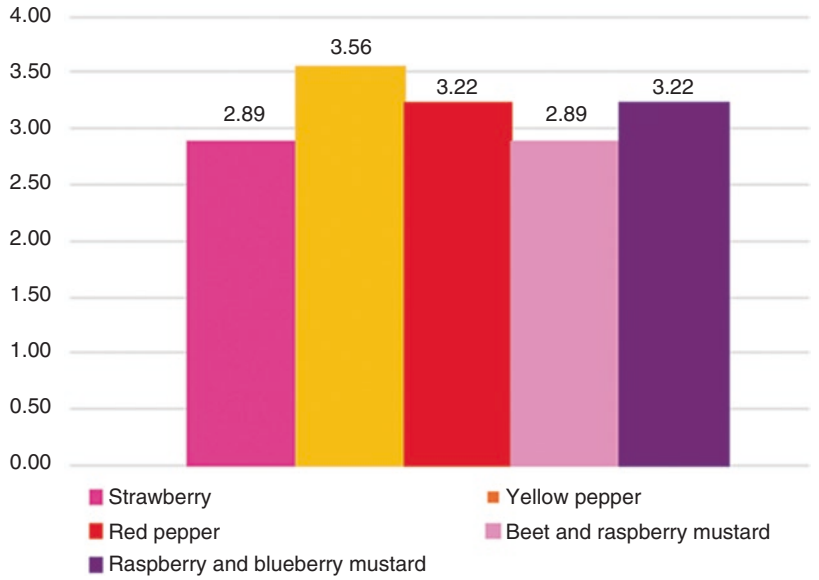

Fig. 5.2 Purchase intention of the samples analyzed by the experts' panel, using a 5-point scale with definite terms between "definitely no" and "certainly yes"

for inclusion in recipes or food pairings depends on a myriad of ingredient characteristics in addition to their flavor profile. Flavor is not necessarily the main role of ingredients, recipes also rely on ingredients to provide the final textures and the overall structure of a given dish (Ahn et al. 2011). Actually, according to the above-mentioned authors, shared flavor compounds represent one of several contributions to the fitness value, while shared compounds clearly play a significant role in some cuisines, other contributions may play a more dominant role in other cuisines. Western cuisines, for example, show a tendency to use pairs of ingredients that share many flavor compounds, supporting the so-called food pairing hypothesis.

5.3.3 Consumer Testing

According to Table 5.2 and overall, the samples were positively appreciated (average > 5), with values ranging from 6.19 ± 1.27 (Red bell pepper smell/odor) to 7.86 ± 1.07 (Raspberry and blueberry mustard color). Regarding appearance, the most prominent was the Raspberry-blueberry mustard cream (7.76 ± 1.15) and the least appreciated was the strawberry emulsion (6.41 ± 1.72). Regarding color, it was again the same Raspberry-blueberry mustard that stood out (7.86 ± 1.07) and the Beet-raspberry mustard was the least appreciated. In the same way, and for the smell/odor parameter, it was the latter that obtained the best results (7.22 ± 1.25) and the red bell pepper emulsion had the lowest result (6.19 ± 1.25). For the most

Table 5.2 Mean values of the parameters analyzed in the study by consumers panel (N = 37), using a a 9-point scale with defined terms situated between "poor" and "excellent

	Strawberry	Yellow pepper	Red pepper	Beet and raspberry mustard	Raspberry and blueberry mustard
Appearance	6.41 ± 1.72	7.49 ± 1.20	7.24 ± 1.40	7.03 ± 1.48	7.76 ± 1.15
Color	6.84 ± 1.50	7.78 ± 1.30	7.08 ± 1.48	6.81 ± 1.39	7.86 ± 1.07
Aroma	6.92 ± 1.68	6.78 ± 1.51	6.19 ± 1.27	7.22 ± 1.25	7.05 ± 1.21
Flavor	6.54 ± 1.43	7.35 ± 1.36	6.73 ± 1.50	7.30 ± 1.31	6.78 ± 1.66
Overall quality	6.65 ± 1.26	7.51 ± 1.06	6.84 ± 1.24	7.22 ± 1.19	7.16 ± 1.39

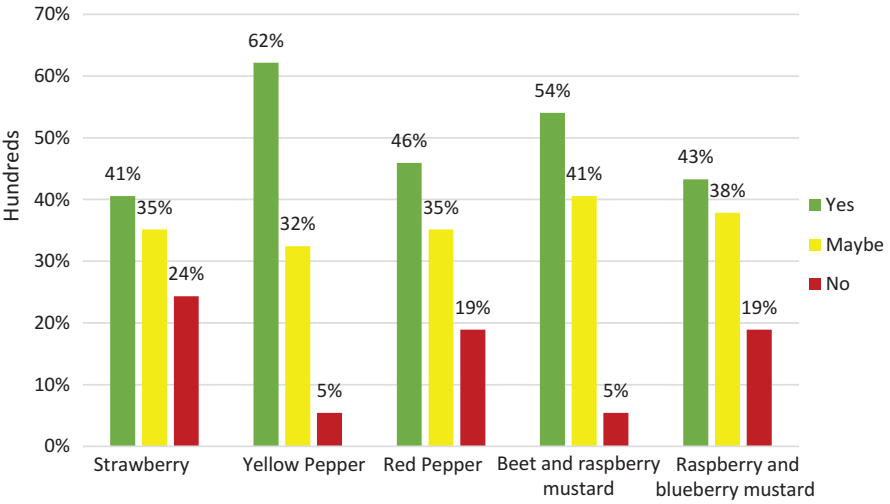

Fig. 5.3 Purchase intention of the samples analyzed by consumers panel, using a 5-point scale with definite terms between "definitely no" and "certainly yes"

flavored flavor/aroma was emulsion yellow bell pepper (7.35 ± 1.36) and the strawberry one was the least appreciated (6.54 ± 1.43).

Regarding the overall appreciation the yellow bell pepper spread stood out (7.51 ± 1.06), and the strawberry spread obtained the lowest result (6.65 ± 1.26) compared to the others.

All samples obtained on average a positive purchase intention (Fig. 5.3). The yellow bell pepper and the beet and raspberry mustard obtained the best marketing potential, 62.2 and 54.10% respectively. The one with the lowest results was strawberry spread, with more divided opinions among the tasters.

The selected and served recipes to the consumers in the tasting menu are indicated in Table 5.3 and correspondent images are showed in Fig. 5.4.

All delicacies developed and presented had positive appreciation (average values of global appreciation between 6.87 and 8.65) (Fig. 5.5).

Table 5.3 Selected ten recipes for the tasting menu

	Name	Emulsion
Couvert		
1.	Yellow pepper dip with potato chips	Yellow pepper
2.	Red pepper dip with king crab meat in ciabatta bread	Red pepper
Entrées		
3.	Pear carpaccio with raspberry, blueberry, honey and lemon vinaigrette, arugula and peanuts	Raspberry and blueberry mustard
4.	Buffalo mozzarella, bacon and dehydrated strawberries, sidra, strawberry and cilantro reduction	Strawberry
Vegetarian		
5.	Red pepper fetuccine, walnuts and basil	Red pepper
Fish		
6.	Cod fillet, fava bean purée and dill	Yellow pepper
7.	Grilled turbot and green asparagus with Sichuan pepper	Beet and raspberry mustard
Meat		
8.	Portuguese beef sandwich with king crab meat	Raspberry and blueberry mustard
9.	Pork tenderloin wrapped in bacon and apricot crust with sweet Potato purée, dehydrated fennel, port reduction and cava mint gel	Beet and raspberry mustard
Dessert		
10.	Black and white in strawberry sauce	Strawberry

The intention to purchase varied for each emulsion and the type of delicacy in which it was used: in some it increased by 57%, but in others it decreased, for

Fig. 5.4 Images of the ten recipes for the tasting menu; numbers correspond to recipes of Table 5.2

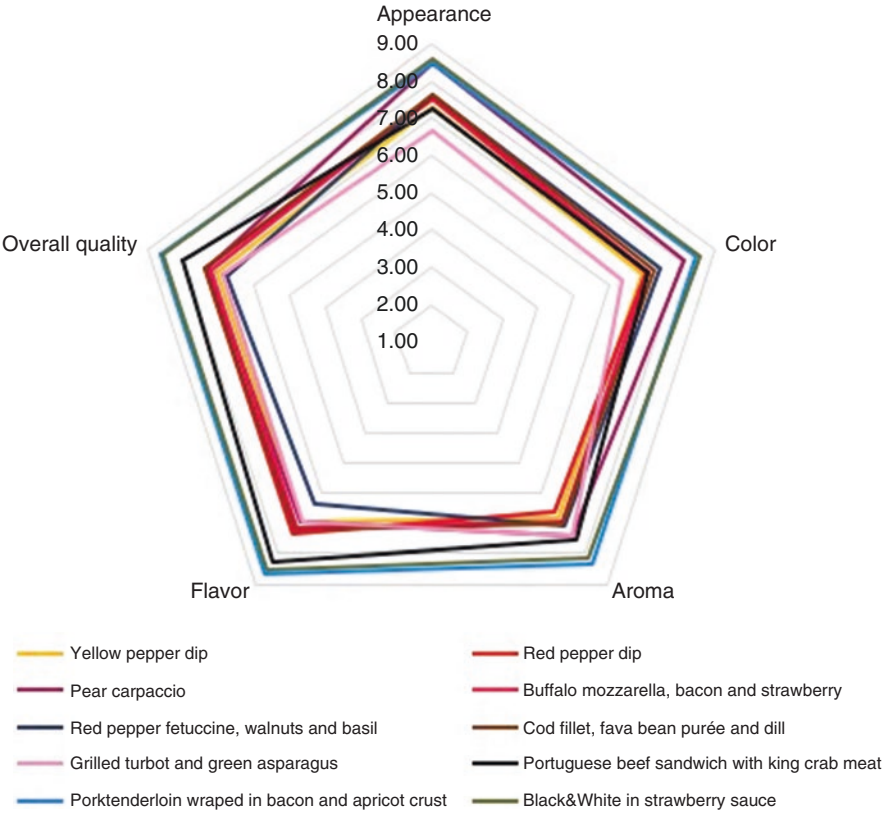

Fig. 5.5 Hedonic sensory evaluation parameters, on average, by dish, analyzed by the consumer tasters, at the technical lunch

example by 14%; globally, most tasters would buy the creams analyzed and see potential commercialization in all emulsions (Fig. 5.6).

Data resulting from this study, demonstrate the importance of revealing recipes or gastronomic applications for new developed products before they are launched on the market so that the potential consumer can find added value, and even accept a premium price, which is distinguished in terms of one or more of the following unique characteristics: quality of ingredients, origin (eg, regional or ethnic), presentation (eg, brand and packaging), composition, raw material, manufacturing process, know-how, availability and a differentiated perception of consumption. (Straete 2008). In this sense, innovation and differentiation based on quality attributes, among which the sensory ones stand out, is extremely important for success.

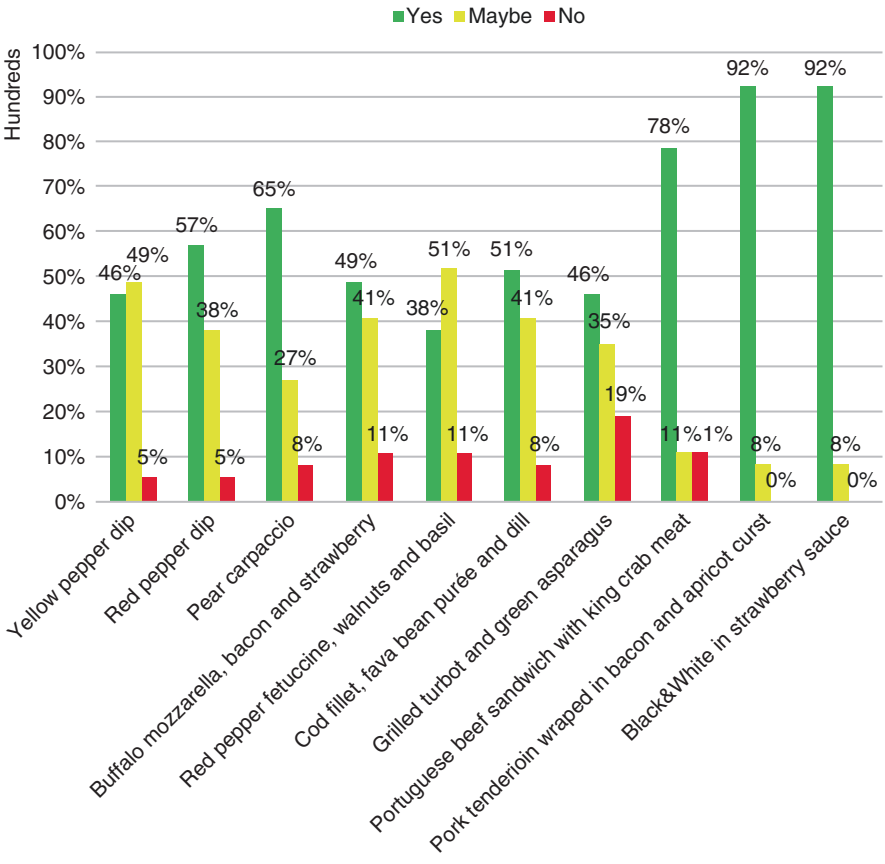

Fig. 5.6 Intention to purchase the emulsions when applied to each delicacy, by the consumer tasters at the technical lunch

5.4 Conclusions

The tested prototypes have potential multiple food applications: pairings; recipes; gastronomic uses. Substantial increase in purchase intention for most samples after tasting the delicacies made with them: most tasters would buy the creams analyzed and see commercialization potential in all emulsions.

The opinion of the chefs was quite important and useful, but not always coincident with the ingredients proposed by the application of Foodpairing®. This tool together with the sensory results and with the available knowledge can be associated with the work of a chef (or other food producer) who demonstrates his experience and ability to generate recipes, techniques and confections, knowing in advance which ingredients will have a higher potential of combination.

Having the possibility of using a panel experienced in culinary arts and food production permitted a broader view on gastronomic possible uses of the tested

products, as these professionals can easily anticipate technical proprieties (both in catering and household environment) and also anticipates the final consumer reactions/acceptance.

Sensory evaluation and consumer testing have shown that the development of culinary applications following product innovation is very important as it may result in the acceptance or not of the product by the end consumer, whether food service or domestic consumer.

Further testing with other types of culinary applications may be made which may provide new evidence for this investigation Nevertheless, given the increasing availability of information on food preparation, this data-driven research has opened new avenues for a systematic understanding of culinary practice, that can be oriented towards a specific food product, like the emulsions and spreads tested.

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Part III
Plant-Based Alternatives to Dairy and Gluten-Based Cereals

Chapter 6
A Technological Optimization to Design a Better Gluten-Free Cereal-Based Cake Premix

Caroline A. Cayres, José L. R. Ascheri, Maria Antonieta P. G. Couto, and Eveline Lopes Almeida

6.1 Introduction

Broadly driven by consumers who voluntarily avoid or limit gluten in their diets, the demand for gluten-free (GF) products continues strong in the foodservice, as well as in retail sectors, and products labeled as GF are showing up more often during in-home meal occasions (Schierhorn 2018). However, the formulation and the manufacture of baked goods without gluten results in considerable technological problems for both cereal technologists and bakers (Gallagher et al. 2004; Matos et al. 2014). The development of food products from other cereals to replace those containing gluten might lead: (1) to attractive aerated products for the section of the population who consumes GF products; and (2) to increase utilization of these cereals for human food, and sorghum is particularly appealing because it can be grown in areas not suitable for wheat (MacRitchie 2010).

Sorghum (*Sorghum bicolor* (L.) Moench), a tropical cereal belonging to the tribe of *Andropogoneae*, is the fifth most important cereal crop worldwide, with more than 57 million tons produced from approximately 41 million ha of land in 2017 (FAOSTAT 2019). It is yet considered of low value to humans and often used as animal feed (Stefoska-Needham et al. 2015). For millions of people in parts of

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Africa and Asia, sorghum is a vital food crop, while it is an underutilized resource in most developed countries (Taylor et al. 2006). Still, it has peaked commercial interests in developed economies on how to make sorghum-based food products due to a growing consumer movement dedicated to "healthy living" (Stefoska-Needham et al. 2015). Nevertheless, unlike wheat proteins, sorghum proteins are not highly functional (Mesa-Stonestreet et al. 2010) because they are encapsulated in protein bodies, and this fact probably turns into unavailable its participation in a viscoelastic dough fibril formation (Duodu et al. 2003). Therefore, a modification of sorghum proteins could be one way to get around the technological properties challenge. In this context, it is mandatory to employ an economical, food-compatible, safe, and scaled up to commercial level method to modify the functional properties of sorghum proteins, to enlarge their applications in food formulations (Mesa-Stonestreet et al. 2010).

As GF has become an expectation for consumers seeking clean label products (Schierhorn 2018), thermoplastic extrusion can be a process used to satisfy this public. The reason is due to its absence of effluents (Guy 2001), and to improve the protein functionality of sorghum (Mesa-Stonestreet et al. 2010), because it is a non-wet cooking process. It is a method in which starch and/or protein and/or dietary fiber are transformed into plastic polymers by adding little water, and they are cooked with a high degree of mechanical energy. The precooking of sorghum flour promoted by thermoplastic extrusion, to produce pregelatinized starch, seems to be effective to reduce the rate of gas loss from the batters, which has an important role in the final cake quality (Taylor et al. 2006). Limited research has been performed on the heat treatment of whole-grain sorghum flour.

Specifically for cakes, reach both oven spring and specific volume similar to a cake with gluten in GF cakes is generally a technological challenge itself, as well as achieving a similar texture. Particle size profile and fiber, protein, and starch contents and types, which impact on hydration and pasting properties, could have a great role in technological characteristics of cakes (Gómez et al. 2008; Wilderjans et al. 2008; Oh et al. 2014; Dhen et al. 2016).

There are many studies, summarized by Di Cairano et al. (2018), showing the importance of the combination of GF flours on the enhancement of dough and biscuits properties, such as technological, nutritional and sensorial characteristics. This relevance can be easily extrapolated to cakes since gluten plays a minor role in both biscuits and cakes. As designing an experiment typically involves selecting among several candidate designs, a very common choice is a mixture design, which is largely used whenever a multi-component system is concerned. In this type of experimental design methodology, the sum of the proportions of the mixture components is always 1, and the other components that are not part of the design must not change. The application of a mixture design can allow finding the optimal composition to achieve a desirable response (Larrosa et al. 2013), using mixture contour plots methodology. Rice offers numerous benefits to be a cereal to take part in a

mixture design, considering its natural bland taste, colorless and hypoallergenic properties (Arslan et al. 2019).

Because of its ready-to-eat nature, availability in many types, and affordable cost, cakes are the most popular bakery items consumed nearly by all levels of society (Jeddou et al. 2017). This fact could explain the reason why there are countless researches focused on the technological quality of GF cakes. On the other hand, studies based on cake premixes are scarce, despite their comparatively good shelf-life. Since consumers do not usually have easy access to GF flours (especially if it is made of an ancient whole-grain) suitable for bakery and confectionery products, it becomes important to offer a carefully industrial prepared cake premix. Therefore, costumers find the convenience of rapidly prepare a GF cake at home.

As the naturally GF flours, such as sorghum, millet, or buckwheat, are not mainstream items in GF foods, because commercial starches are preferably used in these products (Lee et al. 2019), researches into the development of products using GF whole-grains becomes especially important. GF products consumers are progressively demanding GF foods comparable to the long-established gluten ones (Matos et al. 2014). This fact is stimulating much product innovation because GF products do not match with products containing gluten in terms of technological attributes (Arslan et al. 2019). Hence, the present study was focused on optimizing a cake premix formulation, based on GF cereals (sorghum—*in natura* and pregelatinized—and rice) and using orange pomace flour as a fiber-rich coproduct, investigating the technological characteristics of GF batter high ratio type cakes, assessed by the mixture contour plots.

6.2 Material and Methods

6.2.1 Ingredients

The ingredients used in the preparation of the cake premixes and of the cakes were: pregelatinized blend flour (E) (prepared as described in Sect. 6.2.1.2), red sorghum whole-grain (Empório Figueira, São Paulo, Brazil), whey powder (Laticínios Porto Alegre, Ponte Nova, Brazil), vegetal shortening (FRS Alimentos, Barueri, Brazil), alkalized and natural cocoa powders (Pryme Foods, Sorocaba, Brazil), emulsifier (polyglycerol fatty acid esters, mono- and diglycerides of fatty acid and polysorbate 80; Corbion, Araucária, Brazil), sodium acid pyrophosphate and monocalcium phosphate (Diadema Agro-Industrial Ltda, Itapecerica da Serra, Brazil) and chocolate and orange aromas (Frutarom do Brasil Indústria e Comércio Ltda, Porto Feliz, Brazil). Polished rice, wheat flour, corn starch, crystal sugar, salt, sodium bicarbonate, whole milk, egg and margarine were purchased in local markets in the city of Rio de Janeiro (Brazil).

6.2.1.1 Preprocessing of Raw Material

Red sorghum whole-grain flour (S) and polished rice flour (R) were produced through milling these cereals on the disc mill Laboratory Mill 3600 (Perten Instruments AB; Huddinge, Sweden), set to aperture 6.

The by-product of orange juicing (flavedo, albedo, and seed), was ground on a multiprocessor Cadence Mix for You MPR853 (JCS Brasil Eletrodomésticos SA; Balneário Piçarras, Brazil), right after collecting at the food processing industry Bora Bora Comércio e Indústria Ltda (Rio de Janeiro, Brazil), dispersed on trays and dehydrated in a turbo-electric oven FTT 390G (Tedesco; Caxias do Sul, Brazil) at 60 ºC for 5 h. After dehydration, the by-product was milled in a disc mill Laboratory Mill 3600 (Perten Instruments AB; Huddinge, Sweden), set to aperture 6. This product will henceforth be called orange pomace flour.

6.2.1.2 Thermoplastic Extrusion Conditions

S (87.8%), orange pomace flour (7.2%), whey powder (5%), and water (the necessary amount for end conditioning moisture of 16.2%) were mixed on a powder homogenizer Chopin (Tripette & Renaud; Villeneuve la Garenne, France) to prepare E. It was subsequently stored into sealed polyethylene bags, in refrigeration, for 17 h to equilibrate the water distribution. The extrusion was conducted using a single-screw extruder DO-CORDER Brabender (Brabender; Duisburg, Germany). The extrusion profile was: feed section speed: 20 rpm; shear rate: 4:1; screw speed: 160 rpm; feed section temperature: 50 °C; compression section temperature 90 °C; circular die diameter: 3 mm. The extrudate was dehydrated in a fan oven Macanuda Hauber DMS-G (Macanuda Hauber; Joinville, Brazil) at 60 °C for 4 h and subsequently milled on a disc mill Laboratory Mill 3600 (Perten Instruments AB; Huddinge, Sweden), set to aperture 1, and, afterward, on a roller mill Brabender Jr. (Brabender; Duisburg, Germany). The formulation of E and processing conditions of thermoplastic extrusion were established according to the result obtained for an optimization based on better pasting properties of pregelatinized blend flour for cake production employment (unpublished results).

6.2.2 Flours Analyses and Measurements

To obtain a standard flour with gluten suitable for cake production, domestic wheat flour of the market was mixed with corn starch in a 4:1 ratio. The goal of this mixture was to have the protein content approximately 7–8%, as recommended by Yamazaki and Kissell (1978). This flour will henceforth be called reference flour (RF).

Red sorghum whole-grain flour (S), polished rice flour (R), pregelatinized blend flour (E) and reference flour (RF) were characterized by the following analyses:

6.2.2.1 Proximate Composition

The proximate composition was determined according to the official methods of analysis of the Association of Official Analytical Chemists (AOAC 2005), in duplicate measurements: moisture content (Method 925.09), total nitrogen (Method 2001.11, a conversion factor of 5.75 was used to convert total nitrogen to protein content), lipid content (Method 945.38), ash content (Method 923.03), and total dietary fiber (Method 985.29) (analysis without repetitions). Carbohydrates were determined by difference.

6.2.2.2 Particle Size Distribution

The particle size distribution measurement was carried out according to the AACC method n° 66–20.01 (AACC 2010) in a ROTAP sieve shaker RX-29-10 (W.S. Tyler, St. Albans, USA) in duplicate. Seven screen sieve sizes (Newark, USA) were selected (425, 355, 250, 212, 180, 106 and 75μm) and a pan, to obtain a normal distribution of particles from 100 g of sample sieved for 10 min.

6.2.2.3 Hydration Properties

Water solubility index (WSI) and water absorption index (WAI) were determined according to the methodology described by Anderson et al. (1969), in quadruplicate. WSI indicates the amount of sample that is solubilized in water at 30 °C relative to the initial sample, after shaking and centrifuging. In comparison, WAI reports the amount of water absorbed at 30 °C related to the sample that did not solubilize under shaking and centrifugation.

6.2.2.4 Pasting Properties

Viscosity was determined, in an aqueous medium, under heating and stirring, using the equipment *Rapid Visco Analyser* (RVA) (*Newport Scientific*; Warriewood, Australia), according to AACC method n° 76–21.01 (2010), with modifications. The flour was initially stirred during 10 s, at the initial temperature of 25 ºC and 960 rpm. The remainder of the procedure, which lasted 20 min, was conducted at 160 rpm. The initial temperature was held for 2 min and gradually raised, heating to 95 °C, at a constant rate of 14 °C min^{-1}. Then the temperature was held constant for 3 min and cooled to 25 °C, at a constant rate of 14 °C min^{-1}, which was held during the last 5 min of the analysis.

The readings from the pasting curve generated were: pasting temperature (PT), maximum viscosity at 25 °C (MV25), peak viscosity (PV), trough (TR), breakdown (BKD), final viscosity (FV), and setback (SB), obtained through the register realized by the software Thermocline (Newport Scientific; Warriewood, Australia). The measurements were conducted in duplicate.

6.2.2.5 Differential Scanning Calorimetry (DSC)

Starch gelatinization was studied using a calorimeter (TA Instruments, Q200, USA). Indium (In) standards were used to calibrate the energy and temperature of the equipment, and nitrogen was used as the purge gas. Briefly, approximately 3 mg of flour were weighed in hermetic aluminum pans with the aid of a precision scale (Mettler Toledo, Mx5, USA), followed by addition of 6μL of water using a microliter syringe, to maintain hydration. Before the thermal scan, the pans were then hermetically sealed and allowed to equilibrate for 24 h at room temperature. The pans were scanned from 5 to 110 °C at a scanning rate of 10 °C/min using an empty pan as a reference. The determination of the temperatures of onset, peak, and conclusion, as well as the enthalpy of gelatinization, was analyzed by the Universal V4.5A® software (TA Instruments, USA). The measurements were conducted in duplicate.

6.2.3 Cake Premix Production

Preliminary baking tests were made to adjust the cake premix formula. Reference cake premixes were prepared by using the reference flour (RF). The cake premix formula, based on the flour blend weight, was: 120% crystal sugar, 30% vegetal shortening, 10% alkalized cocoa powder, 5% natural cocoa powder, 2.2% sodium bicarbonate, 2% emulsifier, 1.1% sodium acid pyrophosphate, 1.1% monocalcium phosphate, 1% salt, 0.5% chocolate and 0.5% orange aromas. GF cereal-based cake premixes were carried out by replacing flour with gluten with mixtures of S, R, and E according to simplex-centroid mixture design (SCMD), which gave 7 experimental assays (Table 6.1). All variables had the same range (between 0 and 1), and there were no constraints on the design space.

To produce the cake premix, sugar and shortening were mixed during 10 min at medium speed, using a cake batter whip in a planetary mixer (HMT, China). After that, all other ingredients were added and homogenized for 10 min under the same conditions.

6.2.4 Cake Production

Batter high ratio type cakes were made by adding, based on the cake premix weight, 33.3 % whole milk, 33.3 % egg, and 13.3 % margarine to the cake premix (as recommended by commercial cake premixes sold in Brazil), by following an all-in mixing procedure. The aim was to simulate a homemade preparation. They were homogenized in a planetary mixer (HMT, China), using a cake batter whip, for 10 min at medium speed. Subsequently, the batter was poured (45 ± 1 g) for each paper mold which was supported by a stainless cup (7 and 4.5 cm radius at top and bottom, respectively, and 4 cm height). The cup trays containing batter were then baked in an electric oven (Suggar, China) at 170 °C for 20 min. Finally, the cakes

Table 6.1 Results from the assessment of the technological characteristics of the SCMD and reference batters and cakes

Assays	S	R	E	RF	Batter specific gravity[a]	OS[b] (%)	SV[b] (mL/g)	VI[a] (mm)	SI[a] (mm)	UI[a] (mm)	Firmness[c] (N)	Springiness[c] (%)
1	1	0	0		0.78 ± 0.01	55.74 ± 7.44	2.21 ± 0.15	105.0 ± 1.4	0.6 ± 2.4	0.8 ± 0.6	16.79 ± 0.39	44.83 ± 0.32
2	0	1	0		0.86 ± 0.01	91.51 ± 4.72	2.35 ± 0.14	120.3 ± 1.6	6.3 ± 4.4	1.5 ± 0.9	9.91 ± 0.64	48.66 ± 0.49
3	0	0	1		1.00 ± 0.01	46.65 ± 10.53	1.28 ± 0.15	79.6 ± 1.9	3.2 ± 1.4	0.8 ± 0.6	25.49 ± 1.65	41.83 ± 0.48
4	1/2	1/2	0		0.72 ± 0.01	64.06 ± 4.53	2.62 ± 0.08	112.8 ± 2.0	0.3 ± 1.2	0.5 ± 0.3	12.64 ± 0.65	47.81 ± 0.25
5	1/2	0	1/2		0.77 ± 0.01	65.88 ± 6.69	1.84 ± 0.14	99.7 ± 0.9	3.2 ± 1.2	0.8 ± 0.3	19.45 ± 0.87	42.96 ± 0.26
6	0	1/2	1/2		0.77 ± 0.01	72.67 ± 14.84	1.96 ± 0.10	100.6 ± 1.6	3.4 ± 0.7	1 ± 0.6	15.68 ± 1.06	46.02 ± 0.33
7	1/3	1/3	1/3		0.77 ± 0.01	75.21 ± 7.62	2.10 ± 0.11	107.3 ± 1.4	2.0 ± 1.4	0.4 ± 0.4	14.77 ± 0.98	45.53 ± 0.18
8	1/3	1/3	1/3		0.77 ± 0.01	64.47 ± 9.36	2.13 ± 0.12	106.6 ± 2.6	1.9 ± 0.8	0.7 ± 0.9	14.64 ± 0.66	45.44 ± 0.27
9	1/3	1/3	1/3		0.73 ± 0.01	60.50 ± 3.92	2.18 ± 0.08	110.1 ± 1.7	1.1 ± 0.6	0.8 ± 0.1	16.85 ± 0.60	45.71 ± 0.28
10	1/3	1/3	1/3		0.79 ± 0.01	62.42 ± 4.92	2.20 ± 0.08	106.9 ± 1.3	2.4 ± 1.0	0.6 ± 0.4	16.39 ± 0.72	45.76 ± 0.2
Reference				1	0.73 ± 0.01	84.44 ± 5.33	2.41 ± 0.20	130.9 ± 1.3	1.8 ± 0.9	0.8 ± 0.8	5.48 ± 0.20	44.70 ± 0.84

[a]Mean ± standard deviation, n = 3; [b]mean ± standard deviation; n = 6; [c]mean ± standard deviation; n = 10; *S* red sorghum whole-grain flour, *R* polished rice flour, *E* pregelatinized blend flour, *RF* reference flour (gluten-containing), *OS* oven spring, *SV* specific volume, *VI* volume index, *SI* symmetry index, *UI* uniformity index

were removed from the cup and kept at room temperature (25 ± 2 °C) for 1 h before analyses.

6.2.4.1 Batter Specific Gravity

Specific gravity (SG) of cake batter at 25 ± 2 °C was measured by dividing the weight of a certain volume of batter by the weight of an equal volume of water (Matos et al. 2014).

6.2.4.2 Technological Characteristics of the Cakes

Oven Spring
The oven spring (OS) was determined from the difference in the central height of the batter and the central height of the baked cake. A pachymeter was used to obtain these measurements, made in six replicates.

Specific Volume
The specific volume (SV) of the cake was determined by volume/weight. The apparent volume (mL) was measured using the millet seed displacement method according to the AACC method n° 10-05.01 (AACC 2010), and the weight (g) was determined with a semianalytical scale SB12001 (Mettler Toledo, Greifensee, Switzerland). SV was determined in six replicates.

Volume, Symmetry and Uniformity Indices
Volume, symmetry, and uniformity indices were measured according to the AACC approved method n° 10-91.01 (AACC 2010), with template adaptations to the size of cakes (Fig. 6.1). The heights of the cakes were measured with a pachymeter at three points (B, C, and D) along with the cross-sectioned samples. Indices, which were determined in triplicate, were calculated by the Eqs. (6.1)–(6.3).

$$\text{Volume index}\left(\text{VI}\right) = B + C + D \tag{6.1}$$

$$\text{Symmetry index}\left(\text{SI}\right) = 2C - B - D \tag{6.2}$$

$$\text{Uniformity index}\left(\text{UI}\right) = B - D \tag{6.3}$$

Cake Texture
Crumb firmness was determined according to the AACC method n° 74-09.01 (AACC 2010), and an adaptation of this method was used to measure springiness, according to Sangnark and Noomhorm (2004). Texture testing was accomplished on a Texture Analyzer TA.XT Plus (Stable Micro Systems, Surrey, England) and

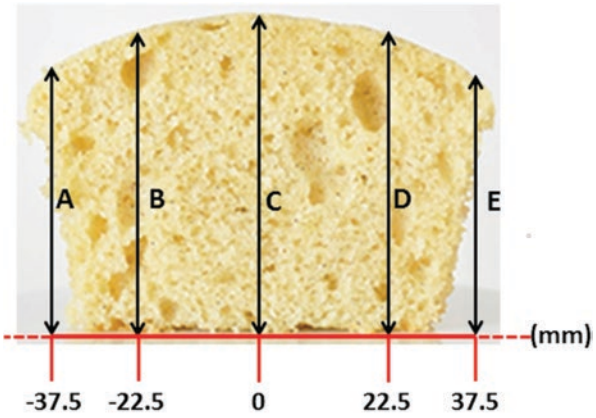

Fig. 6.1 General illustration of standardized cake height measurement points for volume, symmetry, and uniformity indices evaluation for cupcakes. Adapted from AACC International (2010)

fitted with a 30 kg load cell and a P/36 cylindrical aluminum sensor probe. The parameters established were: test option and mode = measurement of the compression force, hold until time; pre-test speed = 2.0 mm/s; test speed = 1.7 mm/s; post-test speed = 10 mm/s; distance = 40% and time = 60 s. In cake texture determinations, the crust was removed by the lowest height of the crust, obtaining a linear surface, and it was assessed the crumb of the sample remainder. Ten replicates were carried out for each cake assay.

Crumb Structure Analysis

To qualitatively evaluate the crumb of cakes, they were cut in half lengthwise. Images were acquired using a flatbed scanner (HP Scanjet G3110, Hewlett-Packard Development Co, Canada). A black box was used to cover the cakes and avoid light contamination.

6.2.5 Quality During Shelf Life

Moisture (M) and water activity (a_w) of the cake premixes were measured on the day they were baked (Day 0), while the specific volume (SV) of the cake was calculated on the Day 0 (the results of the Sect. 6.2.6) and after 180 days (Day 180) of storage of the cake premixes, into sealed polyethylene bags, at 25 ± 2 °C and dark place.

M was determined using Method n° 925.09 of the Association of Official Analytical Chemists (AOAC 2005). a_w was measured in AquaLab LITE (Decagon; Pullman, USA). Both measurements were conducted in duplicate.

6.2.6 Statistical Analyses

Differences between the mean values for the analyses made on flours were assessed by the analysis of variance (ANOVA; $p < 0.05$) and followed by the Tukey test ($p < 0.05$), only when significant differences were observed in ANOVA, using Statistica software 13.0 for Windows (StatSoft Inc.; Tulsa, USA).

The results from the assessment of the technological characteristics of the cakes, as well as the responses of shelf-life study of the cake premixes, were analyzed by the mixture contour plots generated from the fitted regression models obtained by Response Surface Methodology (ANOVA; $p < 0.05$), performed by the same statistical program.

6.2.7 Validation of the Mathematical Models

To validate the fitted regression models, one formulation, which was different from the formulations of the SCMD, was chosen within the ranges studied. It consisted of S = 0.03, R = 0.39 and E = 0.58. Cake premixes and cakes were prepared as described in Sects. 6.2.3 and 6.2.4, and batter specific gravity and technological parameters of the cakes were evaluated following the procedures described in Sects. 6.2.5 and 6.2.6. Subsequently, the experimental values were compared with the predicted values by the single sample Student's t-test (p = 0.05). The analysis of the relative deviation, obtained using Eq. (6.4), was also performed.

$$\text{Relative deviation} = \left| \left(\frac{\text{experimental value} - \text{value predicted by the coded model}}{\text{experimental value}} \right) \right| \times 100 \tag{6.4}$$

6.2.8 Technological Optimization

Numerical optimization is suggested, also using the abovementioned software. Considering the validated models of technological parameters of the cakes, it was employed the desirability function, according to Derringer and Suich (1980), to obtain the best cake in terms of the technological attributes, maximizing the values of OS, SV, VI, SI and springiness and minimizing the values of firmness and UI. The target was maximizing the overall desirability value of the sample.

6.3 Results and Discussion

6.3.1 Batter Specific Gravity

The SG (Table 6.1) is a very important physical property affecting the product quality since it is a measurement of the total air-holding capacity, which is initially incorporated into the batter during the mixing time (Turabi et al. 2008; Zhou et al. 2011). It is important to get proper air incorporation in the batter during mixing because the air bubbles serve as nuclei for other leavening agents (Stauffer 1990). Regrettably, the SG gives little information about bubble size or dispersion (Zhou et al. 2011).

The SG has a direct influence over the final cake volume (Kim and Walker 1992). Low SG is desired in cake batter because it indicates that more air was incorporated into the batter (Turabi et al. 2008), and, therefore, it has been related to a higher cake volume (Gómez et al. 2007). Similarly, the increase in the number of air bubbles in the batter system seems to translate into a tender baked product (Marston et al. 2016), as the change in the density of the batter affects its crumb attributes (Yildiz and Dogan 2014).

For the SG values it was verified, through the mathematical model (Table 6.2), that the increase in S, R and E caused increments in this batter parameter. In contrast, the interaction between R and E displayed a decrease in the SG. It is noticed

Table 6.2 Fitted regression models for the batter specific gravity and the technological parameters of the cakes

Parameters	Fitted regression model
Batter specific gravity (SG)	SG = 0.71 S + 0.83 R + 0.96 E − 0.56 R E [r^2 = 0.7218; p = 0.0419; $p_{(lack\ of\ fit)}$ = 0.0647]
Oven spring (OS) (%)	OS = 56.94 S + 88.27 R + 53.11 E [r^2 = 0.7203; $p_{(model)}$ = 0.0116; $p_{(lack\ of\ fit)}$ = 0.3543]
Specific volume (SV) (mL/g)	SV = 2.24 S + 2.35 R + 1.31 E + 1.32 S R + 0.54 R E [r^2 = 0.9917; p = 2.1825 x 10^{-5}; $p_{(lack\ of\ fit)}$ = 0.2059]
Volume index (VI) (mm)	VI = 105.01 S + 120.53 R + 79.81 E + 29.14 S E + 51.33 S R E [r^2 = 0.9995; p = 2.2582 x 10^{-8}; $p_{(lack\ of\ fit)}$ = 0.3181]
Symmetry index (SI) (mm)	SI = − 0.61 S + 6.29 R + 3.19 E − 9.95 S R + 7.85 S E − 5.15 R E [r^2 = 0.9930; p = 0.0002; $p_{(lack\ of\ fit)}$ = 0.7198]
Uniformity index (UI) (mm)	UI = 0.80 S + 1.44 R + 0.74 E − 2.67 S R [r^2 = 0.9327; p = 0.0007; $p_{(lack\ of\ fit)}$ = 0.5963]
Firmness (N)	Firmness = 15.83 S + 8.82 R + 24.00 E [r^2 = 0.9364; p = 6.5000x10^{-5}; $p_{(lack\ of\ fit)}$ = 0.3226]
Springiness (%)	Springiness = 44.85 S + 48.68 R + 41.85 E + 3.88 S RE − 1.86 S E + 2.72 R E [r^2 = 0.9974; p = 2.9266x10^{-5}; $p_{(lack\ of\ fit)}$ = 0.3654]

S red sorghum whole-grain flour, *R* polished rice flour, *E* pregelatinized blend flour, r^2 coefficient of determination

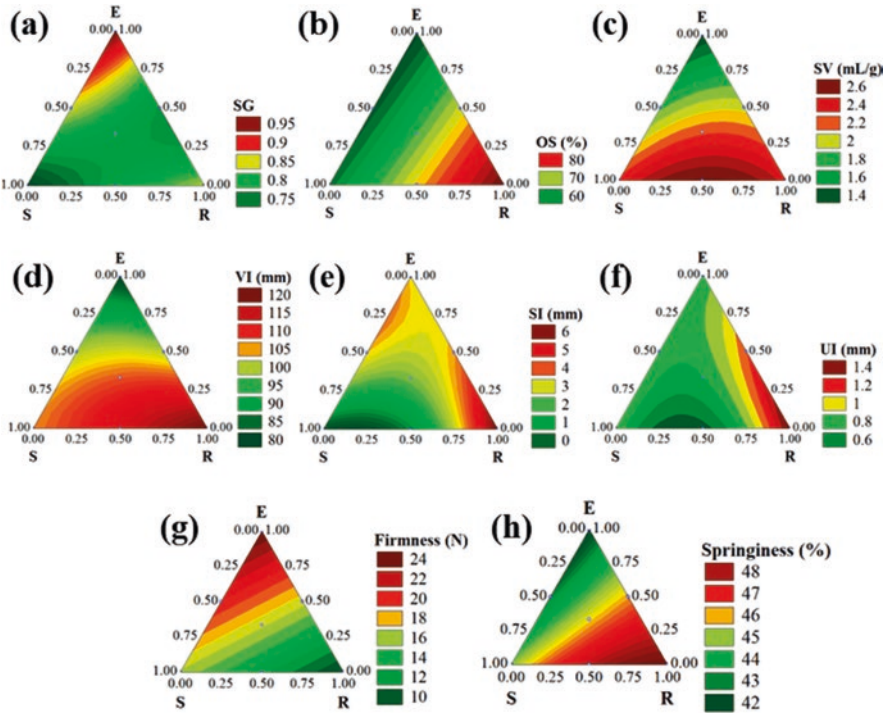

Fig. 6.2 Mixture contour plot for (**a**) specific gravity (SG), (**b**) oven spring (OS), (**c**) specific volume (SV), (**d**) volume index (VI), (**e**) symmetry index (SI), (**f**) uniformity index, (**g**) firmness, and (**h**) springiness. *S* red sorghum whole-grain flour, *R* polished rice flour, *E* pregelatinized blend flour

that the SG was affected more strongly by E variation (Fig. 6.2a). This fact is justified because E is a pregelatinized flour (Fig. 6.3) and contributed as a viscosity increasing agent (thickener-like) at the time of mixing, while S and R contributed to increasing viscosity during baking. Since S is the flour that presented the highest amount of lipids in its composition (Table 6.3), it is plausible that S had shown the highest air-holding capacity during mixing, represented by the lowest coefficient value in the mathematical model (Table 6.2). Lipids contribute to the interface stabilization that favors gas entrapment in the batter matrix (Gularte et al. 2012).

6.3.2 Technological Characteristics of the Cakes

6.3.2.1 Oven Spring

Starch and protein in combination mainly with sugar and fat are fundamental to the structure and other technological properties of bakery products (Hesso et al. 2015). During baking, starch gelatinization, protein denaturation (especially egg protein

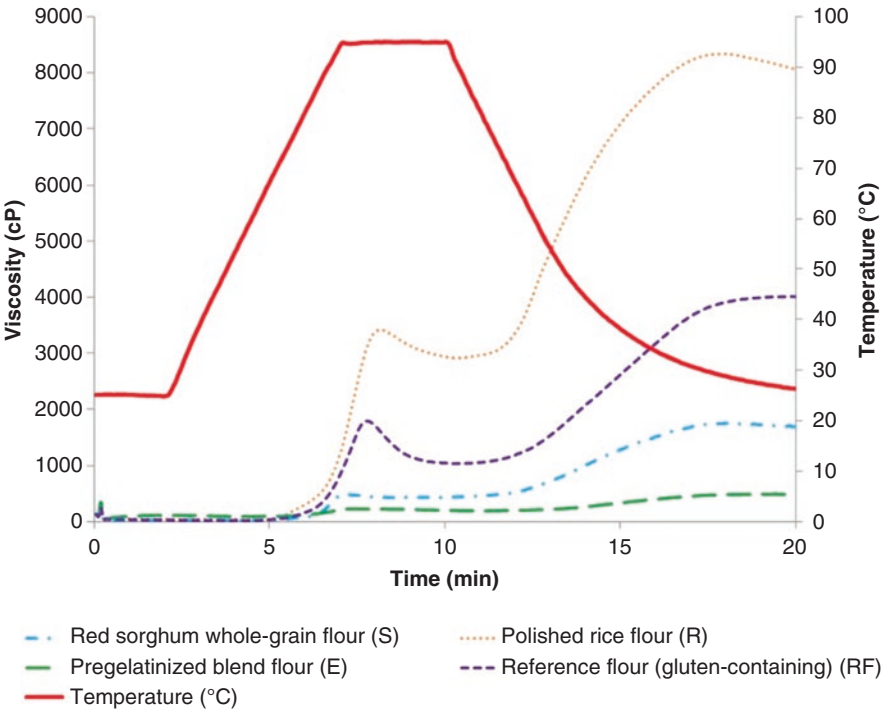

Fig. 6.3 Pasting properties profiles of the flours employed in the cake premixes

coagulation) and air bubbles expansion occur, and cake structure sets as a result of the harmonization of these processes (Yang and Foegeding 2010; Hesso et al. 2015). In the case of chemically leavened cakes, leavening is understood as being due to carbon dioxide (CO_2) produced by chemical reactions, but the expansion of air incorporated into the batter during high-temperature baking also leads the cake to enlarge and form an open interior texture to some extent. Nevertheless, if CO_2 is released too early or too late, the final product has a small height and volume (Stauffer 1990). A more viscous batter with lower SG prevents large air, CO_2 or steam bubbles from coalescing and leaving the batter from the surface (Marston et al. 2016). The baking step underlines the physical and chemical changes in the product components prompting a stabilized crumb structure. During the cooling process, the joined effect of starch gelation and protein coagulation is responsible for the framework setting of the end product (Hesso et al. 2015).

The amount of time available for batter expansion before the structure sets is one important factor for the magnitude of OS (Stauffer 1990). Therefore, as crumb is partially created during baking (Conforti 2014), two important parameters related to the OS are the range of gelatinization temperature and the pasting properties of the flours (Table 6.3 and Fig. 6.3). The starch gelatinization at low temperatures would prevent the correct expansion of batters (Gómez et al. 2008). As higher is the gelatinization temperature, the longer is the development of the crumb (Cauvain and

Table 6.3 Proximate composition, hydration properties, pasting properties and thermal characteristics of the flours used to prepare the cake premixes

	Proximate composition[A] (g/100 g)						Hydration properties[B]	
	Moisture	Protein	Lipids	Ash	Carbohydrates	Total dietary fiber	WSI (%)	WAI (g/g)
S	10.93 ± 0.25 [c]	9.31 ± 0.08 [a]	2.56 ± 0.00 [a]	1.80 ± 0.22 [a]	71.09 ± 0.05 [c]	4.30 ± 0.00 [b]	6.27 ± 0.61 [b]	3.36 ± 0.09 [b]
R	11.95 ± 0.00 [b]	6.73 ± 0.00 [b]	Nd	Nd	81.32 ± 0.00 [a]	Nd	0.47 ± 0.04 [d]	2.81 ± 0.03 [c]
E	6.12 ± 0.03 [d]	9.52 ± 0.04 [a]	1.86 ± 0.01 [b]	1.87 ± 0.01 [a]	70.63 ± 0.04 [c]	9.91 ± 0.00 [a]	10.37 ± 0.32 [a]	4.59 ± 0.12 [a]
RF	12.92 ± 0.05 [a]	7.10 ± 0.02 [b]	Nd	Nd	78.69 ± 0.15 [b]	1.30 ± 0.00 [c]	3.55 ± 0.05 [c]	1.88 ± 0.03 [d]

	Pasting properties[A]							Thermal characteristics[A]			
	PT (°C)	MV25 (cP)	PV (cP)	TR (cP)	BKD (cP)	FV (cP)	SB (cP)	T_o (°C)	T_p (°C)	T_c (°C)	ΔH (J/g)
S	83.6 ± 3.1 [c]	38 ± 1 [c]	489 ± 1 [c]	435 ± 2 [c]	54 ± 1 [c]	1694 ± 1 [c]	1329 ± 26 [c]	65.8 ± 0.9 [a,b]	75.0 ± 0.4 [a]	91.5 ± 0.5 [a]	7.0 ± 1.3 [a]
R	89.2 ± 3.0 [a]	68 ± 6 [b]	3422 ± 0 [a]	2918 ± 1 [a]	504 ± 1 [b]	8066 ± 3 [a]	5423 ± 15 [a]	61.8 ± 0.4 [b,c]	68.6 ± 1.0 [b]	85.4 ± 0.9 [a]	9.9 ± 0.5 [a]
E	75.4 ± 3.0 [d]	116 ± 2 [a]	231 ± 8 [d]	196 ± 6 [d]	35 ± 1 [c]	492 ± 7 [d]	301 ± 4 [d]	68.7 ± 1.9 [a]	76.4 ± 2.6 [a]	91.7 ± 6.3 [a]	1.5 ± 0.3 [b]
RF	85.6 ± 3.1 [b]	49 ± 4 [c,b]	1799 ± 13 [b]	1037 ± 1 [b]	762 ± 12 [a]	4011 ± 14 [b]	2977 ± 16 [b]	60.0 ± 0.0 [c]	65.2 ± 0.1 [b]	85.2 ± 1.7 [a]	8.1 ± 0.2 [a]
									75.4 ± 1.1 [a]		5.4 ± 0.1 [a]

[A]mean ± standard deviation, n = 2; [B]mean ± standard deviation; n = 4; means followed by different superscript letters within each column are significantly different according to Tukey's test ($p < 0.05$); *S* red sorghum whole-grain flour, *R* polished rice flour, *E* pregelatinized blend flour, *RF* reference flour (gluten-containing), *WSI* water solubility index, *WAI* water absorption index, *PT* pasting temperature, *MV25* maximum viscosity at 25 °C, *PV* peak viscosity, *TR* trough, *BKD* breakdown, *FV* final viscosity, *SB* setback, T_o onset temperature, T_p peak temperature, T_c conclusion temperature, ΔH enthalpy change, *Nd* not detectable

Young 2000), which is dependent on the number and size of air, CO_2 or steam bubbles (Conforti 2014). Hence, it is important to have a batter with a suitable viscosity to retain both the air incorporated during mixing and the expanding air nuclei produced by the baking powder during baking, likewise to ensure the uniform dispersion of the ingredients for optimum performance (Wilderjans et al. 2008). In the mathematical model of the OS (Table 6.2), it is showed that increments in S, R, and E increased OS values. Nevertheless, it is observed that R variation affected more intensely the OS than S and E variations (Fig. 6.2b). Meantime, thermal analysis data show that the onset and the peak temperatures of R gelatinization were lower than the S and E ones (Table 6.3). However, the values of PT, PV, TR and BKD of R, are higher than the S and E ones (Table 6.3 and Fig. 6.3), suggesting that, in this study, the viscosity provided by the flours is more important for crumb development than their gelatinization temperatures. Among the three vertex points of SCMD (Fig. 6.2b), only R presented the pasting properties profile suitable to be used alone in a GF cake formulation, to achieve the best OS. S and E must be mixed with another source of starch to reach better values of OS, hence justifying the experimental design adopted in this study. Analyzing the pasting properties profiles of the flours employed in the cake premixes (Fig. 6.3), it can be inferred that the choice of R as another source of starch of SCMD was successful. The aim was to achieve at a GF mixed flour that more closely approached the pasting parameters of the flour with gluten. As R has fewer coarse particles (> 250μm) (Fig. 6.4), this may also

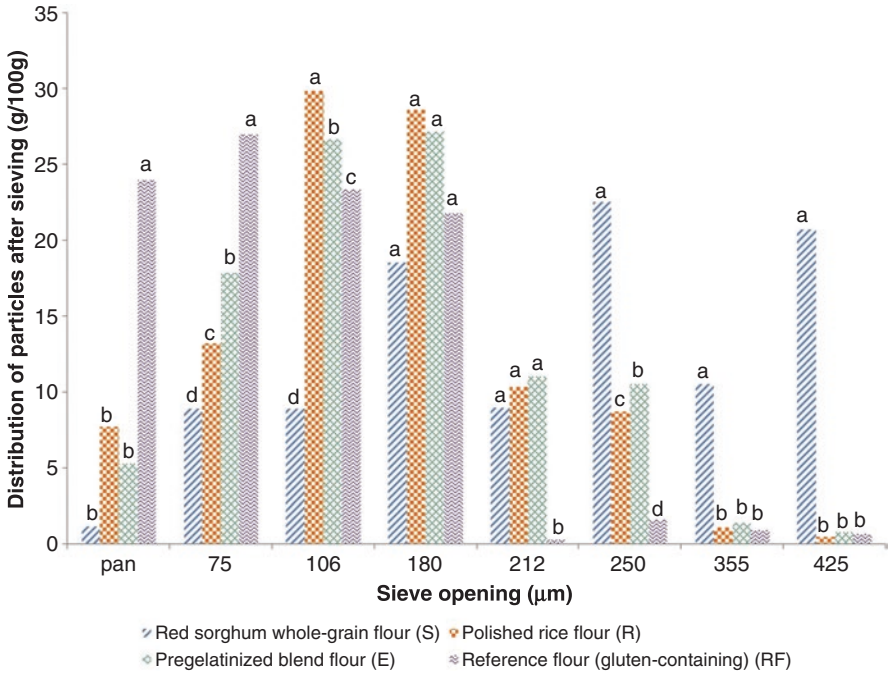

Fig. 6.4 Particle size distribution of the flours used to prepare the cake premixes. Columns with different script letters, within the same sieve opening, are significantly different according to Tukey's test ($p < 0.05$)

have contributed to the higher increase in the OS, because the smaller particles are lighter and therefore easier to be carried by air, CO_2 or steam bubbles. Choi and Baik (2013) stated that small flour particles with a larger total surface area could trap more air bubbles during batter mixing than could coarse particles. This effect was not observed in our results since R did not contribute to decreasing the SG. Nevertheless, R may have contributed to the better bubble distribution, resulting in increased OS. Further investigations would be necessary to corroborate both effects described for the relationship among the flour particle size with the SG and OS.

6.3.3 Specific Volume

Similar to the OS, the mathematical model of the SV (Table 6.2) shows that increases in S, R, E and in the interactions between S and R and R and E caused an increment in the SV. It is noticed that the SV was affected more strongly by R and S variations (Fig. 6.2c). The volume of cake is partly a consequence of the air incorporated in the batter, which agrees with the described results on the SG. Gómez et al. (2008) explained that cake ingredients with higher protein content resulted in a SV value decrease. Among GF flours used, R presented the lowest content of protein and being not significantly different from the flour with gluten (Table 6.3). This result may contribute to diminish the collapse of the structure during the final stages of the baking process or cooling in the cakes with higher amounts of R. Moreover, dietary fiber usually contributes to decreasing the cake volume (Gómez et al. 2010; Itthivadhanapong and Sangnark 2016). This reduction in the volume might be due to the collapse caused by the fibers in the air, CO_2 or steam bubbles during baking (Walker et al. 2014). On the other hand, the increase in the dietary fiber in a cake formulation might increment SV because of its great properties of water absorption and swelling power, which cause high water retention and subsequent increase in SV (Oh et al. 2014). It is also possible that dietary fibers may increase this process temperature by reducing the available water for this process, as well as interacting with starch molecules and, thus, promote cake expansion (Majzoobi et al. 2016). Dietary fibers can also interact with proteins of GF flours and strengthen the overall structure and, therefore, preserve the volume of GF cakes. Further investigations are required to prove these changes. As GF flours used in this study have different dietary fiber content and WAI value (Table 6.3), all of them contribute to the enhancement of the SV. Generally, low SV indicates a dense and less attractive crumb, although high SV does not always mean a desirable cake (Brooker 1993). A cake with high SV may not be associated with a desirable texture, since variations in bubble size and the presence of large bubbles result in an unstable mix, producing cakes of coarse and uneven texture (Conforti 2014). If the bubble does not reach it to the surface, it may instead form a tunnel in the cake (Stauffer 1990), which can generate a cake with high SV, but too porous. For the cake, greater contact and interaction between starch and water resulted in higher volumes (Wilderjans et al. 2008). Due to this, the interaction

between a flour (R), which presented the lowest values for dietary fiber content and WAI, with flours (S and E) that possess higher values of these parameters (Table 6.3) promoted an increase in the SV. It is as if the flours act synergistically to increase the SV, that is, as if there were a compensatory effect of the characteristics abovementioned among R, S, and E. Another feature of the flours that may have contributed to enlarge SV was their particle size distributions because the larger quantities of the flours were in the finer particle size fractions (≤ 180μm) (Fig. 6.1). Changes in volume may be related to differences in air retention and expansion during the baking process (Segundo et al. 2017), in which the size of particles could impact. This result is in agreement with a study of de la Hera et al. (2013), where it was shown that the finest rice flours produced higher volume GF cakes. It is known that the reduction of flour particle size brings about significant improvements in cake quality, e.g., volume and softness, primarily when the flour is used in high ratio cake recipes (Cauvain 2009). Remarkably, most of the cake formulations reached SV values higher than 2.0 mL/g, approaching the reference cake closely, although some assays presented SV values lower than 2.0 mL/g (Table 6.1). This result emphasizes the importance of SCMD in the technological study of GF cereal-base cake formulation.

6.3.4 Volume, Symmetry and Uniformity Indices

As expected, the VI followed a similar tendency as the SV, because the VI is an indicator of cake volume as well. So, the same parameters that influenced the SV influenced the VI too. VI indicates the amount of air entrapped in cake through the cake crumb (Rahmati and Tehrani 2014). The mathematical model of the VI (Table 6.2) shows that increases in S, R, and E caused an increment in the VI. The difference between the SV and VI models was on the interaction factors: they were between S and E and S, R and E, on the VI. This distinction may be related to the different approaches used to measure these responses. Nonetheless, it can be seen in Fig. 6.2 (c and d) that both cake volume measurement responses have similar profiles, that is, they are similarly influenced by the characteristics of R, S, and E discussed above.

The SI shows height differences between central and lateral areas of a cake (De La Hera et al. 2013), expressing how flat the surface of a cake is. If the sum of the heights B and D (laterals) were close to twice the height C (central), then we have a SI value near to zero, indicating a flatter surface. The SI values greater than zero indicate a peak neighboring the center of the surface contour of the cake, while SI values less than zero indicate a collapse on the center of the cake surface contour. This collapse would seem to suggest that the cake structure is not stable enough to support its weight after baking (De La Hera et al. 2013). Consequently, SI is related to gas retention in the final baking phase (Gómez et al. 2008). As it is possible to observe in Table 6.1, all cakes presented SI greater than zero, revealing that the crumb structure of none of them collapsed. It is desirable a the SI greater than zero in batter type cakes, in which they present the "grandma's classic cake format", that

is, a slight peak in the center. In the mathematical model of the SI (Table 6.2), it is verified that increases in R and E caused an increment in SI. In contrast S presented a negative interference in this response. The positive interference of R was greater than that of E (Fig. 6.2e). De la Hera et al. (2013) showed that the finest rice flours produced higher SI in GF cakes. More than 50% of S presented coarse particles (>250μm) (Fig. 6.4) and this may also have contributed to its negative influence in the SI. The larger particles of S may not have been uniformly distributed in the batter during the baking process, thus hindering both the retention of the formed gas and the water vaporization from the mass, decreasing the SI values. Generally, cakes with higher volumes exhibit higher central loaf height (Rahmati and Tehrani 2014), suggesting a causal relationship between the SV and SI. However, this tendency was not observed in this study (Fig. 6.2c, e).

The UI evaluates the distribution of heat inside the oven during the baking process, expressing if the sides of the cakes grew equally. So, the closer the UI value is to zero, the more uniform the contour of the cake. All of the cakes assessed in this study presented satisfactory uniformity (Table 6.1), that is, close to zero. In the mathematical model of the UI (Table 6.2), it is verified that increases in S, R, and E displayed increment in the UI. In contrast, the interaction between S and R displayed a decrease in the UI. Hence, it is possible to notice that the most uniform cakes were those that had higher values of S and R together (Fig. 6.2f).

6.4 Cake Texture

During a product development process, texture analysis is one of the most helpful analytical methods, as it is suitable to quantify the effects of flour blends on the physical properties of the crumb of the bakery products (Jeddou et al. 2017). The texture can be affected by the cake structure (Pizarro et al. 2013), with ingredients' influence the ingredients on the size and distribution of the air cells within the product structure, which consists of air cells distributed throughout its matrix (Sozer et al. 2011). Its tender texture characterizes a desirable cake.

The firmness (Table 6.1) test simulates the gentle squeezing by the hand that consumers apply to bakery products and many other food items (Bourne 1990). The mathematical model of the firmness (Table 6.2) shows that increases in S, R and E caused an increment in firmness. It is noticed that the firmness was affected more strongly by E variation and followed by the S one (Fig. 6.2g). The influence of flours on the firmness was inversely proportional to the OS, SV and VI (Fig. 6.2b, c, d, g), a theoretically expected result, since as higher are the OS, SV and SI, the lower the firmness presented by the cake. This result indicates that the improvement in the softness was due both to an increase in height and cake volume. Springiness (Table 6.1) measures the elastic recovery of a sample and it was determined as a ratio of constant force during time holding to peak force before the holding time (Sangnark and Noomhorm 2004). According to this definition, it is possible to infer that a good cake is one that presents higher springiness values. The mathematical

model of springiness (Table 6.2) shows that increases in S, R, and E and, additionally, in the interactions between S and R and S and E, displayed increment in springiness. It is also showed a negative interference of the interaction between R and E, as it was expected, since the influence of flours on springiness was directly proportional to OS, SV and VI (Fig, 6.2b, c, d, h), while it was inversely proportional to firmness (Fig. 6.2g, h). It is remarkable that: (1) R leaves the cake less firm and more elastic, and that: (2) among the three vertex points of SCMD (Table 6.3), R was the only flour that most approached the firmness of the reference cake. These observations showed the importance of R in this study once again.

6.4.1 Crumb Structure Analysis

The contour, symmetry, and shape of samples can be seen in Fig. 6.5. The quality perceived in a GF cake is considerably related to the appearance of the crumb (Gambuś et al. 2009), as a good cake should show a multitude of evenly distributed minute cells without any large holes (Bennion and Bamford 1997). All the cakes in this study presented good OS, SV and UI, if they are compared with the reference cake, except for the assay 3 in the OS and SV perceived on the images. This

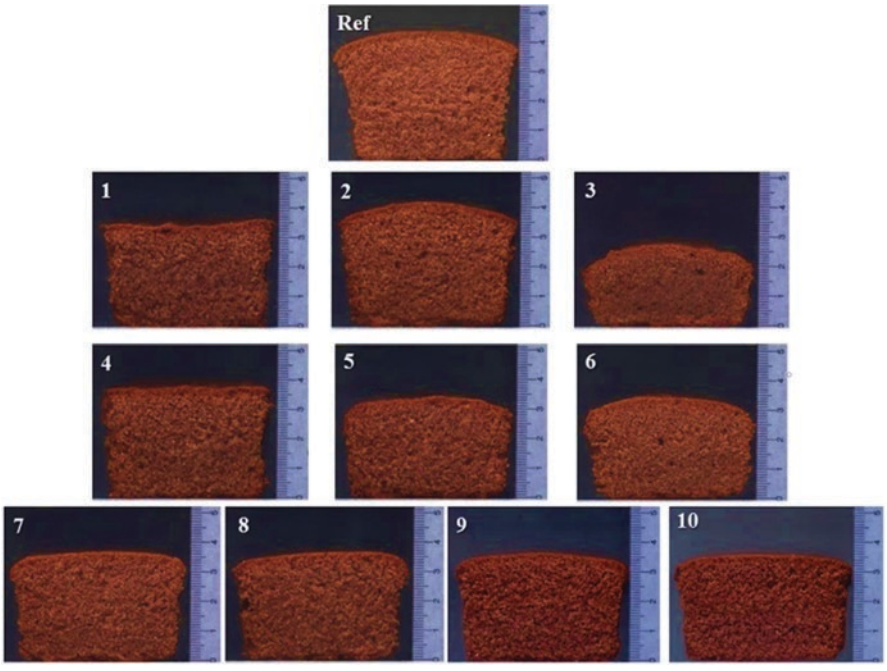

Fig. 6.5 Images of sliced cakes. *Ref* reference. See Table 6.1 for more information about the samples' descriptions

technological problem occurred because the formulation of the cake premix and the ingredients used to prepare the cake itself, i.e., milk, eggs and margarine, were based on industrial formulations which are commercially available already in Brazil market. The amount of liquids added to the premix is practically standardized on the ingredient list of the commercial premixes, and it was chosen to maintain these quantities in this study. Hence, it can be visually concluded that liquids were lacking so that the assay 3 would obtain better technological parameters, considering it is a batter high ratio type cake. Visually, the assay 3 resembles a brownie cake.

6.4.2 Quality During Shelf Life

In a shelf life study of cake premixes, three important parameters should be considered: the M and a_w of the cake premixes themselves and the SV of the cakes. The M and a_w are crucial ingredient characteristics because they will dictate the spoilage speed of a food product. As lower these both parameters are, the lower the pace of microbial activities and chemical reactions in the food matrix. Moreover, the rate of reaction of leavening agents is governed by their rate of dissolution (Stauffer 1990). In this way, the contact with the cake premix moisture already initiates the chemical reaction processes by which the chemical leavening act on bakery products. Cakes prepared from premixes present fluctuation in their quality by the day of their preparation and the day of the premix production. The main reason is that time contributes to decreasing the quality of the final product. Especially for cake premixes, it is important to sustain the capacity to form a cake with the SV as higher as possible, throughout its storage period.

The M varied from 3.16% to 5.16% and a_w ranged from 0.46 and 0.62 (Table 6.4). Mathematical models for the M, a_w and SV-Day180 as a function of S, R, and E

Table 6.4 Results from the assessment of the shelf-life parameters of the SCMD and reference cake premixes

Assays	S	R	E	RF	M[a] (%)	a_w[a]	SV (Day 180)[b] (mL/g)
1	1	0	0		5.1629 ± 0.1039	0.623 ± 0.001	1.83 ± 0.05
2	0	1	0		4.4858 ± 0.0592	0.557 ± 0.003	2.06 ± 0.08
3	0	0	1		3.1585 ± 0.0379	0.463 ± 0.001	1.31 ± 0.10
4	1/2	1/2	0		4.7367 ± 0.1221	0.581 ± 0.001	1.88 ± 0.08
5	1/2	0	1/2		3.9941 ± 0.1134	0.530 ± 0.001	1.71 ± 0.05
6	0	1/2	1/2		3.8175 ± 0.0202	0.525 ± 0.001	1.94 ± 0.09
7	1/3	1/3	1/3		4.2278 ± 0.0769	0.540 ± 0.002	1.95 ± 0.11
8	1/3	1/3	1/3		4.3096 ± 0.0232	0.549 ± 0.001	1.93 ± 0.08
9	1/3	1/3	1/3		4.3136 ± 0.0267	0.541 ± 0.001	1.96 ± 0.06
10	1/3	1/3	1/3		4.3890 ± 0.0775	0.541 ± 0.001	1.94 ± 0.06
Reference				1	2.0380 ± 0.1132	0.561 ± 0.001	2.52 ± 0.05

[a]Mean ± standard deviation, n = 2; [b]mean ± standard deviation; n = 6; *S* red sorghum whole-grain flour, *R* polished rice flour, *E* pregelatinized blend flour, *RF* reference flour (gluten-containing), *M* moisture, a_w water activity, *SV* specific volume

Table 6.5 Fitted regression models for the shelf-life parameters of the cake premixes

Parameters	Fitted regression model
Moisture (M) (%)	$M = 5.16\ S + 4.48\ R + 3.16\ E - 0.35\ S\ R - 0.66\ S\ E + 4.17\ S\ R\ E$ [$r^2 = 0.9950$; $p = 0.0001$; $p_{(lack\ of\ fit)} = 0.9583$]
Water activity (a_w)	$a_w = 0.62\ S + 0.56\ R + 0.46\ E - 0.04\ S\ R - 0.05\ S\ E + 0.06\ R\ E$ [$r^2 = 0.9951$; $p = 0.0001$; $p_{(lack\ of\ fit)} = 1.0000$]
Specific volume Day 180 (SV-Day180) (mL/g)	$SV\text{-}Day180 = 1.83\ S + 2.06\ R + 1.31\ E - 0.26\ S\ R + 0.56\ S\ E + 1.02\ R\ E + 1.76\ S\ R\ E$ [$r^2 = 0.9988$; $p = 0.0002$; $p_{(lack\ of\ fit)} = -$]

S red sorghum whole-grain flour, *R* polished rice flour, *E* pregelatinized blend flour, r^2 coefficient of determination

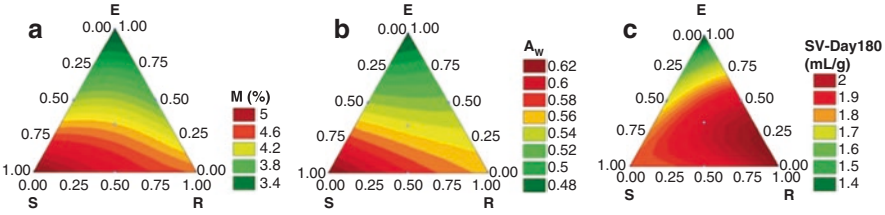

Fig. 6.6 Mixture contour plot for (**a**) moisture (M), (**b**) water activity (a_w), and (**c**) specific volume of the gluten-free cakes after 180 days of storage (SV-Day180) of the cake premixes. *S* red sorghum whole-grain flour, *R* polished rice flour, *E* pregelatinized blend flour

were found and showed interaction effects (Table 6.5). In the M and a_w, the related impacts to E were the lowest. Hence, an increment in E is the least contributing to increase these responses (Fig. 6.6a, b), which is desirable to heighten shelf-life related to microbiological, enzymatic, or chemical activities.

When comparing the SV in Day 0 (Fig. 6.2c) with the SV after 180 days of cake premixes storage (Fig. 6.6c), through mixture contour plots it is concluded that: (1) in Day 0, an increment in any range of E entails a decrease of SV; (2) in Day 180, an increment of until 50% of E contributes to maintaining SV in the highest values region. Thus, to retain food safety and technological aspects of these cake premixes, E was shown to be a possible option. Complementarily, Karaoğlu et al. (2001) found that pregelatinized starch extends shelf life to the cakes by delaying the staling process.

6.4.3 Validation of the Mathematical Models

It can be seen that the experimental values for the SG, OS, SV, VI, SI, UI, firmness and springiness and these values predicted by the models did not present a significant difference in the single sample Student's t-test ($p > 0.05$) (Table 6.6). Therefore, we may consider as validated the models for the SG, OS, SV, VI, SI, UI, firmness and springiness.

Table 6.6 Experimental values and the values predicted by the fitted regression models for the batter specific gravity and the technological parameters of the cakes

	Experimental values	Values predicted by the fitted regression models	Relative deviation
Batter specific gravity (SG)	0.75 ± 0.03[a]	0.73[a]	2.33%
Oven spring (OS) (%)	64.29 ± 6.42[a]	66.94[a]	4.12%
Specific volume (SV) (mL/g)	1.91 ± 0.08[a]	1.88[a]	1.51%
Volume index (VI) (mm)	97.9 ± 0.8[a]	97.3[a]	0.63%
Symmetry index (SI) (mm)	2.8 ± 0.4[a]	3.1 [a]	10.40%
Uniformity index (UI) (mm)	0.6 ± 0.7[a]	1.0[a]	73.57%
Firmness (N)	17.62 ± 0.79[a]	17.83 [a]	1.22%
Springiness (%)	45.41 ± 0.31[a]	45.25 [a]	0.35%

[a]Means followed by the same letter on a row are statistically equal at 5% significance according to single sample Student's t-test

6.4.4 Technological Optimization

At the established conditions for the optimization, the optimal flour formulation for the cake premixes was: S = 20% and R = 80%, with a desirability value of 0.516 (Fig. 6.7). Despite not having any E, this optimized formulation is one way to insert sorghum in industrialized foodstuffs, considering the growing demand for GF, more sustainable, convenient and high-quality foods.

6.5 Conclusions

R was shown to be an important cereal in this study, as it improved many of the technological properties of cake (OS, SV, VI, SI, UI, firmness, and springiness) discussed here. Therefore, if the main objective is the introduction of an unconventional cereal, such as sorghum, in industrialized food products, the use of R is suggested to increase the technological quality of GF cereal-based cakes. Regardless of this fact, it is important to highlight that the use of a higher amount of liquid ingredients in the cake preparation could improve the technological characteristics of the products in which there were more S and E.

One optimized technological formulation for GF cake premixes have been suggested, in which the optimal flour formulation for the cake premixes was S = 20% and R = 80%. Although S and E have presented worse technological yields than R, they have great potential in cake production. It is essential to carry future studies that increase the amount of liquids in the formulations, for example, mainly because it was proved that E extends the shelf-life of the cake premix. These technological

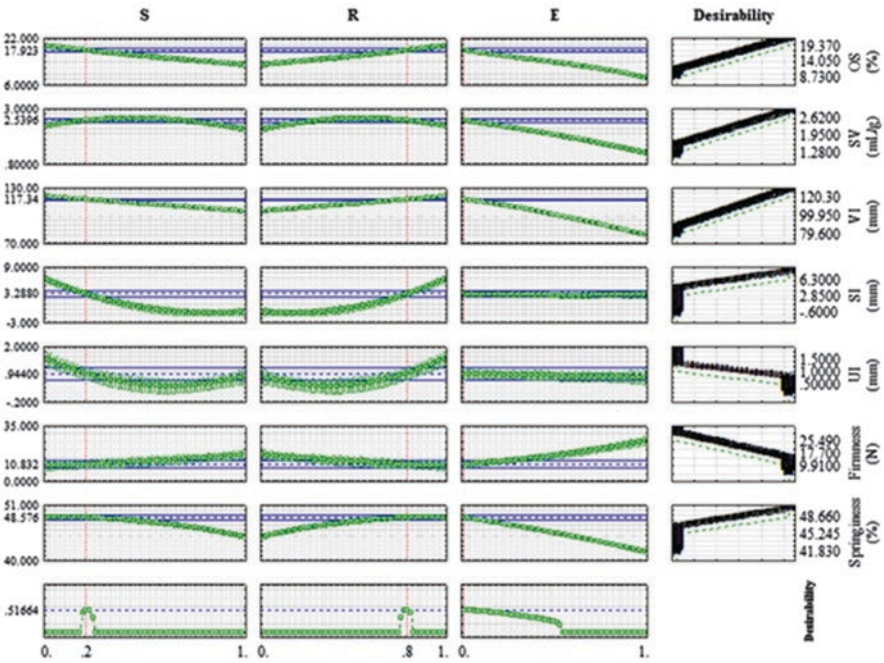

Fig. 6.7 Profile of predicted values and desirability for the technological optimization

evidences could also act as a catalyst for the uptake and demand for sorghum by the food industry and consumers.

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Chapter 7
Effect of Partial Replacement of Milk Protein by Vegetable Proteins on the Texture of *Requeijão*

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7.1 Introduction

According to the Food and Agricultural Organization of the United Nations (FAO 2016), the dried seeds obtained from leguminous plants are denominated pulses. Singh (2017) remarks that all pulses are considered leguminous, but not all leguminous are considered pulses. The (FAO 2016) states that pulses include kidney beans, lima beans, butter beans and broad beans. Chickpeas, cowpeas, black-eyed peas and pigeon peas are also pulses, as well as all varieties of lentils. Pulses have been considered by several researchers as the second source of human food, being surpassed only by cereals. Their importance is due to the high content of macronutrients such as proteins and carbohydrates; micronutrients such as vitamins and minerals and their low calories and lipid content (Asif et al. 2013; Roy et al. 2010). Their bioactive constituents have been attracting attention and raising the interest of the food industry. Studies report benefits such as reducing blood plasma glucose levels, which aids in the control of obesity, diabetes, lowering serum cholesterol levels, and preventing cardiovascular disease. Furthermore, the peptides found in pulses may present antioxidant, antimicrobial, and antitumor activity (Mudryj et al. 2014).

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7.1.1 Composition

Regarding macronutrients, pulses contain approximately 21–25% protein, almost twice the content found in cereals (Singh 2017). However, they have limited levels of essential amino acids such as methionine, tryptophan and cysteine (Singh 2017; Havemeier et al. 2017). Therefore, it is recommended that pulses are consumed in combination with other sources of vegetal or animal protein to form a good quality protein blend and complement the diet (Asif et al. 2013; Havemeier et al. 2017). The protein content and amino acid composition vary according to the variety, germination, environment and application of fertilizers. Globulins, stock proteins rich in glutamine, aspartic acid, arginine and lysine, correspond to 37–72% of the protein composition. Legumins and vicilins, rich in methionine and cysteine, are the main globulins found in pulse seeds. The complementary fraction consists of albumin, which contributes approximately 15–25% of the total protein of the seeds. This fraction is rich in cysteine, methionine and lysine (Boye et al. 2010a, 2010b; Singh 2017; Roy et al. 2010). According to some authors (Boye et al. 2010a, 2010b; Singh 2017), pulse proteins present poor digestibility, which may limit their application to food formulations. The digestibility of the protein varies according to the characteristics of starch present in the seed, which causes it to increase (Singh 2017). Other authors, such as Havemeier et al. (2017), however, report that pulses are considered good sources of digestible proteins. Nosworthy and House (2017), in turn, report that the methods used to determine the protein quality should be considered when assessing it, since the preparation method may alter the quality of the protein.

Carbohydrates correspond to approximately 70% of the composition of pulses. They can be either hydrolyzable by gastrointestinal enzymes, absorbed and metabolized, or those that are unavailable for not being hydrolyzed by digestive enzymes. Starch corresponds to the largest fraction of pulses and is subdivided into amylose and amylopectin. A large variation in the amylose content in pulse starch has been reported. For being rich in complex carbohydrates, pulses have a low glycemic index as they slowly release glucose into the bloodstream. Furthermore, they stimulate prebiotic activity, which is beneficial to health. Bioactive carbohydrates include dietary fibers that include cellulose, hemicellulose, gums, pectins, mucilages, β-glucans, lignins, resistant starch, undigestible oligosaccharides, among others. These substances resist the digestion process and absorption in the small intestine of humans, with partial or complete fermentation in the large intestine. Concerning pulse starch, research demonstrates a strong tendency towards retrogradation and difficulty in swelling and breaking of grains during cooking in comparison to cereal starch. The high fiber content in pulses may increase by satiety (Havemeier et al. 2017; Singh et al. 2017; Tosh and Yada 2010).

Regarding micronutrients, pulses are sources of thiamine, niacin, folate, riboflavin, pyridoxine, potassium, vitamin E, vitamin A, selenium, iron, zinc, calcium and magnesium (Singh 2017; Havemeier et al. 2017; Asif et al. 2013). Recent studies demonstrate that pulses are also rich in polyphenols, tannins, alkaloids, saponins, oxalates, phytates, lectins (hemagglutinins) and enzyme inhibitors (mainly trypsin

and chymotrypsin), compounds often considered as antinutritional (Roy et al. 2010). The concentrations of these compounds vary according to the species and variety. Seed coatings, for example, are rich in water-insoluble fibers and polyphenols, while cotyledons are rich in soluble fibers, slow-digesting oligosaccharides and resistant starch (Singh et al. 2017; Mudryj et al. 2014).

Phenolic compounds interfere with protein digestibility in the human organism, leading to low amino acid bioavailability. Tannins, which act as a defense mechanism in plants, have a chelating capacity with metal ions and form hydrogen bonds with proteins, reducing mineral absorption and protein digestibility. They form complexes with starch or its digestive enzymes, as well as conferring astringency sensory characteristics. Phytic acid forms irreversible complexes with proteins and minerals (Roy et al. 2010; Singh et al. 2017).

Despite their antinutritional characteristics, research has suggested that these substances, usually concentrated in seed husks, can prevent several diseases (McCrory et al. 2010; Mudryj et al. 2014). Polyphenols present high therapeutic potential due to their antioxidant activity. Phenolic acids and their derivatives (flavonols, anthocyanins, condensed tannins) are the main categories of polyphenols found in the seeds of leguminous. They act as primary antioxidants by donating hydrogen atoms to free radicals. Its antioxidant action helps prevent the oxidation of lipids, proteins and DNA by species reactive to oxygen which are produced in the cells during oxidation. Ferulic acid is the most abundant phenolic compound followed by p-coumaric acid and sinapic acid. Flavonoid glycosides, anthocyanins and tannins add color to the seed (McCrory et al. 2010; Asif et al. 2013; Mudryj et al. 2014; Singh et al. 2017).

Phytosterols are sterols similar to cholesterol in structure. They have been largely evaluated as functional ingredients for their beneficial health effects, such as reducing cholesterol levels and reducing the intestinal absorption of dietary or endogenous cholesterol. The major phytosterols present in pulses include sitosterol, stigmasterol and campesterol (Havemeier et al. 2017; Singh et al. 2017).

7.1.2 Extraction Processes

Pulses can be processed by using different techniques for producing flour, concentrates and isolates. One of the techniques employed involves spiral airflow classification after grinding, which results in the fractionation of the grains/seeds into high starch flours and high protein flours. The grinding process results in particles that can be separated by size and density. The thinnest fraction corresponds to the protein fraction while the coarser corresponds to the fraction rich in starch (Boye et al. 2010a, 2010b; Sozer et al. 2017).

Alkaline solubilization followed by isoelectric precipitation is a widely used technique for the extraction of proteins from pulses. This technique is based on the solubility of pulse proteins at a higher pH (between 8 and 11) and their low solubility at a pH close to the isoelectric point (4.5). After the isoelectric precipitation, the

protein is separated by centrifugation, washed, neutralized and dried. Another technique utilized is the acid extraction of proteins from pulses, which is also based on solubilization, but in an acidic medium (pH lower than 4) with subsequent isoelectric precipitation, cryoprecipitation or membrane separation (Boye et al. 2010a, 2010b).

Pulse proteins can also be extracted in water without further precipitation stages. Successive extractions with a subsequent centrifugation stage are required for a higher yield. Salting-in and salting-out techniques may be employed for protein extraction from pulses. These techniques are based on the ionic strength of saline solutions for the precipitation of proteins, which are subsequently separated by centrifugation (Boye et al. 2010a, 2010b).

Another possibility is the membrane separation, which can be employed as a complement to the isoelectric precipitation. The extracts obtained by acid or alkaline precipitation are concentrated by ultrafiltration processes utilizing membranes selected for the retention of proteins with specific molecular masses (Boye et al. 2010a, 2010b).

7.1.3 Functional Properties and Applications

Due to their nutraceutical and functional properties, pulses are considered promising for composing ingredients in the form of concentrates or isolates of high acceptability and low cost. The industrial application of pulse proteins depends on the physicochemical and structural properties as well as on the conditions of the process (Toews and Wang 2013). Globulins are soluble in saline solutions while albumins are soluble in water. Prolamines and glutenins, which appear in smaller fractions, are soluble in alcohol and basic/acid medium, respectively (Shevkani et al. 2019).

The functional properties of major relevance to the industrial application include the water solubility, emulsifying capacity, water and lipid absorption, foaming and gelling behavior. Such properties vary according to differences in the molecular characteristics of the proteins, such as amino acid composition, molecular mass, secondary structure, charge distribution, hydrophobicity, among others. Albumin has very important characteristics as it interacts and competes with starch due to its solubility in water (Boye et al. 2010a, 2010b; Shevkani et al. 2019).

The solubility is associated with the properties of emulsification, foaming and gelling. From a technological point of view, solubility depends on the balance between hydrophilicity and hydrophobicity of the protein structure. The pH of the medium exerts a strong influence on the solubility of the protein (Boye et al. 2010a, 2010b; Shevkani et al. 2019).

Emulsions consisting of two immiscible liquids in suspension or dispersion form a system of low stability due to interfacial surface tension. To avoid coalescence, it is necessary to promote the stability of the system, which can be achieved by using proteins that form elastic and cohesive layers around the oil droplets. The stability

of the emulsion is achieved by the ability of the proteins to increase the viscosity of the continuous phase and to decrease the movement rate of the oil droplets in the system. The emulsifying power has been considered an important property of the pulsed proteins for mass application in meat products, ice cream, soups, mayonnaise among other products (Boye et al. 2010a, 2010b, Shevkani et al. 2019).

Food texture and aroma retention are associated with the capacity of proteins of absorbing lipids and water. Water absorption capacity is important for products such as soups, pasta, creams, bakery products. This capacity is related to the hydrophilic fraction of the protein chains. Fat absorption is important for products such as ground meals, meat substitutes, extenders, baked foods. They are related to the buccal sensation and retention of aromas. These properties are associated with the ability of the nonpolar amino acid fraction to bind to the aliphatic chains of oils and fats. The application of pulses in gluten-free products has been explored due to its viscoelastic capacity in the production of bread and pasta (Boye et al. 2010a, 2010b; Sozer et al. 2017).

Bean curds may also be obtained from pulse proteins, resulting in products similar to tofu, which is soy-based. The foaming capacity is bound to the ability of the proteins to diffuse into the interfaces, reorient themselves and form a viscous film without excessive aggregation. The stability depends on the ability of the protein to maintain the foam. This property is very important for products that require aeration, such as souffles, mousses, ice cream, toppings, among others. The gelling property is important for products such as creams, soups, puddings, meat products among others. This property is obtained when proteins are subjected to heat, pressure and ionic forces (Boye et al. 2010a, 2010b, Shevkani et al. 2019).

Another productive sector of interest for the application of pulses is that of meat products, to obtain texture and yield properties, such as hamburger, sausages, nuggets, and the like (Boye et al. 2010a, 2010b, Shevkani et al. 2019).

Another promising application is the encapsulation for protection of the bioactive and volatile components such as omega 3 fatty acids, phytosterols and carotenoids (Can Karaka et al. 2015). Extruded products such as breakfast cereals, snacks, nut-like products, croutons, crackers and wafer can also be produced (Asif et al. 2013; Sozer et al. 2017).

On the other hand, pulses may give off-taste due to the presence of phenolic compounds and alkaloids. However, this limitation may be corrected or eliminated during processing. In processing, the thermal degradation of the phenolic acids and tamine and the formation of products of Maillard reaction during heating (reaction between amino acids and reducing sugar) may lead to the formation of foreign odors. Oxidation reactions in conjunction with the thermal degradation of carotenoids may also contribute to the formation of foreign odors, as well as degradation of unsaturated fatty acids by the action of lipoxygenases (Roland et al. 2017).

7.1.4 Use of Pulses in Dairy Products

There is little information on the use of pulses as an ingredient for dairy products. Pulse proteins may find applications for products similar to dairy due to their fiber and starch content. However, it is still limited (Asif et al. 2013; Zare et al. 2012).

Cow milk is one of the main raw materials used for producing cheese. However, other sources of milk may be used to produce certain types of cheese, such as the Roquefort which is made from sheep milk. Goat milk is widely used in the production of Chabichou and Sainte-Maure cheese. Buffalo milk, in turn, is widely used for the production of mozzarella cheese.

The *requeijão*, a typically Brazilian product, is classified as fused cheese. It is produced by grinding the mass of any type of cheese (however, depending on the type of cheese utilized, the structure and especially the sensory aspects of the final product may be quite distinct), and then mixing it with some ingredients such as fat, milk powder, whey and emulsifying salts such as polyphosphates. The most prominent product in the Brazilian market is creamy *requeijão* (Cunha et al. 2012; Oliveira et al. 2016). There are different types of *requeijão*, for which the humidity, fat and protein contents vary according to the region and mainly to the manufacturer. The different technologies employed result in products with different rheological characteristics, such as spreadability and texture (Salek et al. 2015; El-Garhi et al. 2018).

The basic composition of a *requeijão* is 58–60% humidity, 1–2% carbohydrate, 1–1.5% sodium chloride, 24–27% fat and 9–11% protein. And presenting 60 to 62% of fat in the dry extract and pH between 5.7 and 6.2. pH has a great influence on the consistency of fused cheeses, especially for *requeijão*, where there is a specific pH range that maintains its past-like form. *Requeijões* with pH from 5.5 to 5.7 have a firm consistency while those with a pH of around 4.9 are drier and those with a pH close 6.5 are softer. (Perry 2004; Van Dender 2014).

The aim in this chapter was to evaluate the potential of pulses in the form of high protein and low starch concentrates as promising ingredients in the partial replacement of animal protein by vegetal proteins in the production of creamy cheese.

7.2 Material and Methods

7.2.1 Formulations of Requeijões

In order to establish an initial formulation for this study, a market research was conducted, in which the nutritional composition, based on the label data, of 20 different brands of *requeijão* present in the Brazilian market were evaluated (Table 7.1).

The amount of the ingredients to be used in the formulations of this study was calculated based upon the average composition obtained from the commercial formulations and the requirements of the Brazilian legislation for *requeijão* (Table 7.2).

Table 7.1 Nutrition facts of some of the leading *requeijões* commonly available in supermarkets

	Nutrition facts (portion—100 g)		
Brand[a]	Carbohydrate	Protein	Total fat
A	3.00	9.00	25.00
B	0.00	7.00	27.00
C	3.33	10.33	25.00
D	2.00	9.00	30.00
E	4.00	10.33	21.00
F	6.00	6.66	20.66
G	2.00	9.70	26.00
H	4.66	4.66	21.66
I	4.66	9.33	24.00
J	3.00	9.66	24.66
K	2.33	10.66	21.00
L	2.66	10.00	24.00
M	2.00	9.33	27.30
N	0.00	10.00	23.66
O	0.00	6.67	26.67
P	0.00	12.00	24.66
Q	0.00	10.00	23.00
R	2.33	10.66	21.00
S	4.33	10.00	30.00
T	1.66	10.66	22.66
Average	(2.40 ± 1.79)	(9.28 ± 1.75)	(24.45 ± 2.80)

Source: Authors, 2019

[a]The brand names are substituted for letters

Finally, from preliminary tests, the replacement of dairy protein by vegetal proteins (pulses) was defined as 25% and 3 pulses (fava bean, pea and lentil) were evaluated, whose proportions varied in each formulation according to levels established by using a simplex factorial mixture design (Table 7.3).

The Geiger equipment, model UMMSK-12E, which consists of a jacketed cooking pan, was used for the production of the *requeijão*. From its total capacity of 12 kg of production, the minimum quantity required for it to function, which is 5 kg, was utilized. The jacketed pan has a homogenizing shovel and cutting knife located inside the bowl. The equipment operates with both direct and indirect steam. To avoid the incorporation of water into the product during manufacture, the indirect steam option was used in this study. The steps adopted for the formulation of the *requeijão* (adapted from Van Dender 2014) are schematically described below.

Cold grinding the mass in Geiger equipment at high rotation for 3 min.

Cold homogenization of the mixture at high rotation (mass, melting salt and NaCl) for 1 min.

Addition of the half part of water and milk cream.

First cooking—heating of the mass under stirring (70–75 °C for 5 min).

Table 7.2 List of the ingredients utilized in the creamy cheese formulations

Ingredients	(%)
Mineral water	18–21
Cheese mass	17–20
Cream	51–54
Modified corn starch	1–4
Milk protein concentrate	2–5
Refined salt	0.5–1.5
Melting salt	0.5–1.5
Fava protein	0–4
Pea protein	0–4
Lentil protein	0–4
Potassium Sorbate	0.10–0.40
Creamy cheese aroma	0.02–0.10
Lactic acid	0.10–0.40

Source: Authors, 2019

Addition of the remaining ingredients (dairy compound, vegetable protein, potassium sorbate, acids and water).

Second cooking—heating of the mass under stirring (90 °C for 3 min).

Hot jar filling (70–75 °C).

Cooling/Storage (5 °C).

After the production of the formulations, the product was packed in two types of packaging. The part of the product intended for the analysis of viscosity was packed in clear glass jars with a capacity of approximately 210 g, a total height of 103 mm, an outside diameter of 72 mm and a mass of 245 g. The jars were closed with conventional screw-on lids.

The other part of the formulations, intended for the texture and physicochemical analysis, was packed in transparent, low-density polyethylene containers with a total capacity of 200 g, a total height of 92 mm, a diameter of 55 mm at the top margin and 64 mm at the base, with a mass of 3.8 g. An aluminium lid was utilized for this container, which was subsequently fixed by heating, with the aid of manual sealing equipment, Huhtamaki brand, model 111—AD series. Then, a simple snap-on traditional lid was used. Figure 7.1 shows the packaging used for placing the products.

7.2.1.1 Texture Analyses

A Stabela Micro System texturometer TA.XT plus was utilized for the texture analysis. Recently prepared *requeijão* samples (maximum 3 days), which were stored in a refrigerator (8–10 °C), were used. Each sample was analyzed in six repetitions.

Table 7.3 Factorial mixture design to evaluate the percentage of substitution of dairy protein by vegetal protein (pulses) in the requeijão formulation and its effects on the texture parameters

Assay	Fava (%)	Pea (%)	Lentil (%)	Firmness (N)	Shear stress (N.s)	Spreadability (N)	Adhesiveness (N)	Viscosity (Pa.s). 10^2
1	25 (1)	0 (0)	0 (0)	9.74	6.56	−13.09	−2.42	7.29
2	0 (0)	25 (1)	0 (0)	7.72	5.43	−10.50	−1.79	6.14
3	0 (0)	0 (0)	25 (1)	8.84	6.42	−11.70	−2.31	7.53
4	12.5 (1/2)	12.5 (1/2)	0 (0)	6.64	4.43	−9.24	−1.47	5.31
5	12.5 (1/2)	0 (0)	12.5 (1/2)	7.90	5.56	−10.56	−1.84	6.85
6	0 (0)	12.5 (1/2)	12.5 (1/2)	8.63	6.27	−11.41	−2.11	7.62
7	16.6 (2/3)	4.2 (1/6)	4.2 (1/6)	7.78	5.46	−10.50	−1.71	6.76
8	4.2 (1/6)	16.6 (2/3)	4.2 (1/6)	7.88	5.62	−10.64	−1.77	6.15
9	4.2 (1/6)	4.2 (1/6)	16.6 (2/3)	7.57	5.28	−10.34	−1.73	6.45
10	8.3 (1/3)	8.3 (1/3)	8.3 (1/3)	7.37	5.02	−10.20	−1.64	6.42
11	8.3 (1/3)	8.3 (1/3)	8.3 (1/3)	7.75	5.29	−10.71	−1.75	6.75
12	8.3 (1/3)	8.3 (1/3)	8.3 (1/3)	8.47	5.83	−10.88	−1.95	6.93
Control[a]	–	–	–	5.75	3.28	−8.77	−1.25	4.32

Source: Authors, 2019

[a]Control = *requeijão* without the addition of pulses

All analyses were performed in six replicates and the coefficient of variation (CV) ranged: firmness: from 1.5 to 7.2%; shear stress: from 2.9 to 8.6%; spreadability: from 1.7 to 6.1%; adhesiveness: from 5.4 to 16.9; viscosity: from 0.4 to 14.7%

For each analysis, approximately 10 g of the sample, with a temperature previously set to 20 ° C, was placed in a female cone and pressed to eliminate air pockets. The excess sample was removed with a knife until the surface was flat.

After the preparation of the sample, the female cone was placed in the texturometer holder and fitted to the male cone. The two cones were aligned and the measurement started by penetrating the probe contained in the male cone into the sample. The probe penetrated the sample by 23 mm and then returned to the surface. The force required for the probe to reach the maximum depth (23 mm) was defined as the firmness of the sample; the application area of the penetration force was defined as shear stress. When the probe was removed from the sample, the force applied defined the spreadability and the negative area of the application of force, the adhesiveness. Figure 7.2 illustrates the functioning of the texturometer for the

Fig. 7.1 Picture of creamy cheese packages. Source: Authors, 2019

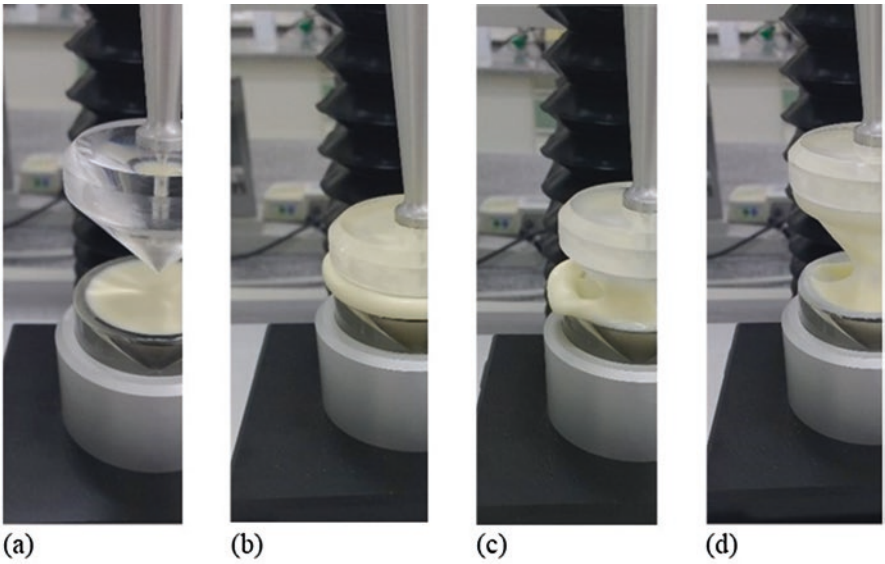

Fig. 7.2 Texture analysis steps of *requeijão* samples, being (**a**) and (**b**) probe penetration steps to measure firmness and shear stress and (**c**) and (**d**) probe removal to measure spreadability and adhesiveness . Source: Authors, 2019

measurements performed. The procedure followed the instructions provided by the texturometer manufacturer.

The analysis of viscosity was conducted with samples stored in glass flasks three days after processing. A Brookfield viscometer model RVDV-II + Pro containing a

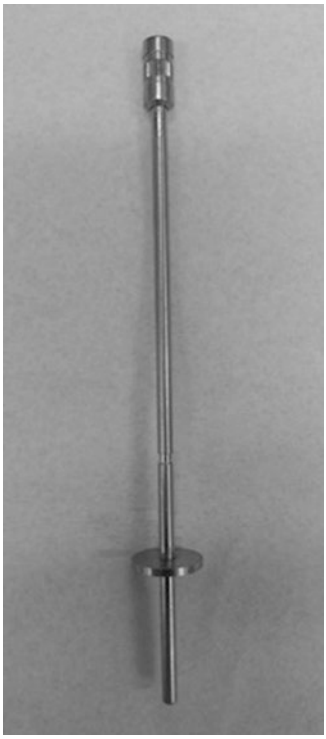

Fig. 7.3 Spindle number 6 applied to measure viscosity of *requeijão* samples. Source: Authors, 2019

spindle of number 6 (as shown in Fig. 7.3) was used. 10 rpm rotation speed and 50% torque were applied. The temperature of the samples was maintained between 10 °C and 12 °C during the analysis.

7.3 Physicochemical Analyses

The physicochemical analyses applied to the *requeijão* formulation were: pH, humidity, color (L, *a*, *b*), protein and lipids. The pH was measured by direct reading of the pH of the samples at room temperature using bench pH meter. The analysis of humidity analysis was performed utilizing the oven method, drying the samples at 105 °C, at atmospheric pressure, until the samples achieved constant weight. The color (L, *a*, *b*) was measured utilizing a spectrophotometer (Konical Minolta, model CM-2600D). The protein analysis was performed by acid digestion of samples followed by reaction with concentrated sodium hydroxide, sample distillation, collection of nitrogen fraction in boric acid solution and titration with hydrochloric acid, according to Kjeldahl method. Lipid analysis was performed by acid digestion of the sample in the presence of isoamyl alcohol, according to Gerber method (Zenebon and Pascuet 2008).

7.4 Results and Discussion

From the data displayed in Table 7.1, the *requeijão* cheese formulation was defined for this study containing 9–10% protein, 24–26% lipids and 3–5% carbohydrates. These macronutrient concentration ranges were used to calculate the concentration ranges of each ingredient to be added in the formulations as shown in Table 7.2.

Table 7.3 presents the quantities of each type of pulse used for each formulation of *requeijão*. The percentages indicated refer to the substitution made concerning the milk protein. In all assays, the substitution was 25% of milk protein for vegetable protein, whose ratio/type of vegetable protein is variable in each test. The answers obtained for the parameters of texture are also presented in Table 7.3. In addition to the texture analyses, physicochemical analyses of the *requeijão* formulations were performed, whose results can be seen in Table 7.4.

Table 7.4 Factorial mixture design to evaluate the percentage of substitution of dairy protein by vegetal protein in the *requeijão* formulation and its effects on the physicochemical parameters

Assay	Fava (%)	Pea (%)	Lentil (%)	pH	Humidity (% w/w)	Protein (%w/w)	Lipid (% w/w)	L	a	b
1	25 (1)	0 (0)	0 (0)	5.93	59.06	9.06	25.35	84.41	−0.71	19.16
2	0 (0)	25 (1)	0 (0)	5.84	59.29	9.39	25.52	84.50	0.71	22.10
3	0 (0)	0 (0)	25 (1)	5.84	58.95	9.41	22.95	84.45	0.17	19.65
4	12.5 (1/2)	12.5 (1/2)	0 (0)	5.84	59.33	9.74	25.87	84.36	0.05	20.36
5	12.5 (1/2)	0 (0)	12.5 (1/2)	5.86	59.43	9.19	24.79	84.47	−0.21	19.03
6	0 (0)	12.5 (1/2)	12.5 (1/2)	5.83	58.82	9.90	26.06	86.10	0.51	20.41
7	16.6 (2/3)	4.2 (1/6)	4.2 (1/6)	5.85	59.35	9.53	26.56	83.92	−0.30	18.75
8	4.2 (1/6)	16.6 (2/3)	4.2 (1/6)	5.77	59.18	9.28	25.04	84.18	0.33	20.39
9	4.2 (1/6)	4.2 (1/6)	16.6 (2/3)	5.80	59.35	9.56	25.19	83.07	0.04	19.04
10	8.3 (1/3)	8.3 (1/3)	8.3 (1/3)	5.82	59.44	9.65	24.02	84.00	0.05	19.57
11	8.3 (1/3)	8.3 (1/3)	8.3 (1/3)	5.86	59.38	9.30	22.67	84.77	0.13	19.34
12	8.3 (1/3)	8.3 (1/3)	8.3 (1/3)	5.84	59.29	9.47	26.00	84.46	0.09	19.37
Control[a]	–	–	–	5.93	59.77	9.80	22.18	85.48	−0.94	15.59

Source: Authors, 2019

[a]Control = *requeijão* without the addition of pulses

All analyses were performed in three replicates and the coefficient of variation (CV) ranged: pH: from 0.1 to 0.4%; humidity: from 0.02 to 0.76%; protein: from 0.2 to 6.8%; lipid: from 0.4 to 6.7; L: from 0.4 to 14.7%; a: from 6.1 to 269.5% and b: from 1.0 to 2.2%

Color *a* presented high values of CV in samples that average were very close to zero

For each texture (Table 7.3) and physicochemical (Table 7.4) parameter, linear mathematical models (with and without interactions) were evaluated to explain the variation observed of the properties of the *requeijão*. The results can be seen in Table 7.5. A response surface analysis (Fig. 7.3) was also performed for each model that was considered statistically significant and predictive. The models with high reliability ($p < 0.05$) and high correlation (correlation coefficient greater than 0.85) were considered.

All texture responses presented an increase concerning the control formulation which contained dairy protein only (the increases compared to the control were: from 15.5 to 74.9% for firmness, from 34.6 to 99.7% for shear stress, from 5.3 to 49.2% for spreadability and from 18.1 to 93.7% for adhesiveness). However, the effect of each pulse was different in each response evaluated. A greater increase in the value of the variables firmness and shear stress responses was observed as a function of the increase in the concentrations of fava bean and lentil, whereas the replacement by pea caused a less significant increase in these parameters. There was a negative interaction between fava bean x pea and fava bean x lentil, which indicates that pulse mixture formulations were less firm and with have less shear stress than those that used pulses in isolation.

Regarding the spreadability and adhesiveness, as the parameters present a negative sign, the analysis was made according to the modulus of these responses. Thus, the results observed presented the same tendency of the first two variables, that is,

Table 7.5 Mathematical models for texture and physicochemical parameters for *requeijão* with partial replacement of milk protein by vegetal proteins (fava beans, peas and lentils)

Parameter	Linear model equation[a]	*p*-value	R^2	R^2 adjusted	R
Firmness	**FI = 9.62 F +8.02 P +8.84 L −6.90 (F.P) −4.71 (F.L)**	0.03	0.75	0.60	0.87
Shear stress	**SH = 6.54 F +5.69 P +6.40 L −5.39 (F.P) −3.43 (F.L)**	0.03	0.75	0.60	0.87
Spreadability	**SP = −12.91 F −10.83 P −11.73 L +8.40 (F. P) +6.15 (F.L)**	<0.01	0.83	0.73	0.91
Adhesiveness	**ST = −2.39 F −1.85 P −2.28 L +2.28 (F.P) +2.02 (F.L)**	<0.01	0.84	0.74	0.92
Viscosity	**V = 7.19 F +6.14 P +7.12 L −5.03 (F.P) +3.09 (P.L)**	0.02	0.77	0.64	0.88
pH	pH = 5.90 F +5.80 P +5.82 L	0.04	0.50	0.39	0.71
Humidity	**H = 59.03 F +59.25 P +58.98 L +0.89 (F.P) +2.11 (F.L) −0.81 (P.L)**	0.02	0.85	0.72	0.92
Protein	P = 9.23 F +9.61 P +9.53 L	0.39	0.19	0.01	0.44
Lipid	LP = 25.07 F +23.57 P +25.56 L	0.51	0.14	0.00	0.37
L	*L* = 84.05 F +84.74 P +84.38 L	0.72	0.07	0.00	0.26
Color *a*	***a* = −0.68 F +0.72 P +0.18 L**	<0.01	0.99	0.98	0.99
Color *b*	***b* = 18.95 F +21.59 P +19.40 L −3.05 (F.L)**	<0.01	0.87	0.82	0.93

[a]In all equations fava beans, peas and lentils are represented by letters F, P and L, respectively
Models marked in bold are considered statistically significant and predictive

the increase of the fava bean and lentil concentrations promoted a greater increase of spreadability and adhesiveness and there was a negative interaction (decrease of these parameters) in the formulations with the pulse combinations fava beans x peas and fava beans x lentils.

The viscosity, in turn, presented a different behavior, and the highest values for this parameter were obtained by the formulations with higher lentil concentration and the interactions of the variables occurred in the opposite direction. While there was a negative interaction (viscosity reduction) between fava bean x pea, a positive interaction (increased viscosity) between lentil x pea was observed.

The two main findings of the present study were that: (1) the partial substitution of dairy protein by vegetal protein caused an increase in the values of all the parameters related to texture evaluated, that is, all texture parameters were higher in the formulations containing pulses than in the control condition that used milk protein only (Table 7.3); (2) the use of fava bean and lentil proved to increase texture parameters more than the use of pea.

The functional properties of the *requeijão* depend on several factors such as the characteristics of the milk proteins, fat and melting salts content, processing conditions as well as melting temperature and the pH of the final product. However, in this study, all these conditions were maintained constant and the only variations between formulations concern the type and concentration of the pulses utilized. Therefore, the increases in the texture parameters must be related to the use of vegetal proteins.

According to Boye et al. (2010a, 2010b), the amino acids that appear most in pulses are glutamine, aspartic acid, arginine, lysine, methionine and cysteine, which are all polar amino acids (except for methionine). On the other hand, Fox et al. (1998) state that the main amino acids present in caseins are valine, leucine, isoleucine, phenylalanine, tryptophan and proline, which are nonpolar amino acids. In this way, the partial replacement of dairy protein by proteins from pulses promotes an increase in the content of polar amino acid and, consequently, an increase in the hydrophilic character of the proteins present in the *requeijão*, increasing the protein-water interaction, which helps to explain the increase in the texture parameters of the product.

The differences in the effects of each pulse may also be related to the specific composition of each of them, the pulse-pulse and pulse-casein interaction, and the formation of protein co-aggregates (pulse-pulse and pulse-casein) that were three-dimensional networks of variable stability. All these factors must be better studied for a complete understanding of the action of pulses as ingredients in dairy products such as *requeijão*.

There are few studies in the literature comparing the different types of pulse, especially in dairy products. Fernández-Quintela et al. (1997) reported similarity in the capacity of water absorption of fava bean and pea pulses. However, this author verified that the fava pulse had a greater capacity of fat absorption than the pea pulse. Such observation may help explain the lower values of texture parameters in formulations with higher pea concentration. The lower interaction of the pea pulse with fat leaves this component freer in the medium and it is well known that fat is a

factor that promotes the reduction of viscosity in *requeijão* (Van Dender 2014). A similarity between the emulsifying power of fava, pea and lentil pulses was also observed in other studies (Boye et al. 2010a, 2010b; Shevkani et al. 2019). Such similarity may support the hypothesis that the differences between products with and without the addition of pulses must be related to the interaction of the pulses with milk protein and the other ingredients of the formulation.

The statistical evaluation for pH indicated that the interactions between pulses were not significant and that although the model (Table 7.5) presented p-value less than 0.05, the determination and correlation coefficients were very low. Therefore, the model obtained was not considered statistically significant and predictive. The pH values were all very similar (5.77–5.93), which can be explained by the use of lactic acid as an acidity regulator. The values obtained in this study agree with the pH value range in commercial *requeijão* sold in the Brazilian market, which generally ranges from 5.7 to 6.0 (Van Dender 2014).

The humidity analysis also indicated very similar values between the different formulations. However, there was considerable positive interaction between the fava bean x lentil which increased the humidity by two percentage points on average. The statistical analysis indicates that there was a synergy between these two pulses in water retention in the product, which may help explain why there was a negative interaction between these variables in the texture parameters. A greater quantity of water in the final product is a factor that can lead to decreased firmness, shear stress, spreadability and adhesiveness.

According to da Silva et al. (2012), the increase in the humidity content in light cheese is a factor that compensates for the reduction in fat content, since it promotes the dissolution of the protein matrix, compensating for the increase in viscosity caused by fat reduction. Similarly, in this study, the addition of pulses caused an increase in the texture parameters, thus, the higher final humidity content may exert the same effect verified in light cheeses, that is, to promote the dissolution of the protein matrix reducing the values of the texture parameters.

Due to the standardization of ingredients adopted in this study, the protein and lipid contents of all formulations were very similar and, therefore, no statistically significant models were obtained for these physicochemical parameters.

Regarding color, the parameter L (luminosity) did not present a statistically significant model. Table 7.4 indicates that the results were practically the same for all formulations. For the parameter a (red/green coordinate), however, large differences were observed in the use of each pulse: increasing the concentration of the fava pulse increased the green color whereas increasing the concentration of the pea pulse increased the color red. This difference must be assessed carefully from an industrial point of view as it can have a major influence on the sensory acceptance of consumers. Comparing the color of the formulations with the control, the control was the greenest of all ($a = -0.94$) and the addition of the pulses altered the color of the product increasing the red color, being the fava bean pulse the least influencing for this response and the pea pulse was the one that changed the color of the formulations the most.

As for parameter b (yellow/blue coordinate), the coefficients of all pulses were positive and higher than the control formulation, indicating that pulse use intensified the yellow coloration of the product. The formulations with pea pulse presented the highest intensity of the yellow color by the response surface analysis. However, there was a negative interaction between the fava bean and pea, that is, the association of these two pulses left the color yellow less bright, which is also an issue to be observed from the sensory point of view.

There is a tendency of *requeijão* formulations to have a whiter and less yellow coloration in the Brazilian market generally. The increase in yellow color may be a negative factor for the sensory acceptance of the product. In the present study, the average increase of the yellow color of *requeijão* produced utilizing pulses was 26.8% compared with the control formulation. This difference may have an impact on the sensorial acceptance of *requeijões* contained pulses. Cunha et al. (2010) evaluated the production of *requeijão* with substitution of dairy fat by hydrogenated vegetal fat (25 and 50% substitution) and found that the formulation with 50% fat substitution was the one with the lowest yellow coloration (parameter b) and was also the one with the highest sensory acceptance of color in tests with consumers.

7.5 Conclusion

The study presented in this chapter represents a contribution to the literature on the application of pulses in dairy products. The data obtained demonstrated that the substitution of 25% of milk protein for fava bean, pea and/or lentil pulses is possible for the production of *requeijão* (a typical Brazilian cream cheese) with characteristics similar to those of the product obtained with milk protein only, in the process conditions utilized.

The partial substitution of milk protein for pulses had a greater impact on the texture, which increased all texture parameters analyzed (firmness, shear stress, spreadability, adhesiveness and viscosity). The most probable causes for these alterations are the increased amount of polar amino acids (which affects the interaction of the protein matrix with the water and fat of the product), the formation of protein co-aggregates, and pulse-pulse, pulse-casein interactions and pulse interactions with other ingredients of the formulation. The processing conditions such as pH, ionic strength, temperature and agitation also contribute to denaturation and alteration of the proteins, influencing the final results.

The type of pulse utilized has an impact on the variation of the final properties of the product. The fava bean pulse caused the greatest alterations in texture whereas the pea pulse changed the color of the formulations significantly compared to the control formulation. On the other hand, the use of mixtures of pulses presented few interactions, being the most relevant those related to the final humidity of the product, in which the interaction of the pulses of fava bean and lentil increased while the interaction between the pulses of pea and lentil decreased it. The use of the pulses had an impact on the final cost of the product, with a reduction of cost depending on

the concentration and type of pulse utilized, which is promising in the food industry. Finally, the advancement in studies of substitution of animal raw materials for vegetal ones may promote impacts regarding sustainability in food production and nutritional issues for consumers, which are relevant subjects for further research.

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Part IV
Consumer Behavior

Chapter 8
Evaluation of Consumers' Acceptance of Bread Supplemented with Insect Protein

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8.1 Introduction

8.1.1 Need for Novel Food Sources

In the next decades, the world population will reach 9 billion, which will result in a significant increase in food production, especially in animal-derived protein (Boland et al. 2013). This increase is also caused by globalization, as developing countries are adopting Western dietary habits, which are richer in the consumption of meat (Msangi and Rosegrant 2011).

This rise in meat production will exacerbate some of the livestock sector impacts on the environment—namely greenhouse gas emissions and atmospheric ammonia emissions (Gerber et al. 2013; Steinfeld et al. 2006)—and also consumer health (Aykan 2015).

With these concerns regarding the current and future sustainability of the production and consumption of meat, there is a growing urgency to change the alimentary habits to follow others that are more environmentally and economically sustainable (Burlingame and Dernini 2012). Strategies that only reduce the meat production or that just lead to slight changes in diets (greater consumption of eggs, vegetables or poultry) will only attenuate the problems and not fix them (Eisler et al. 2014). One of the solutions that have been gaining interest is the possibility of utilizing novel food sources (insects, algae, underutilized pulses) that not only allow

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economic/environmental sustainability but also fulfill nutritional requirements (van der Spiegel et al. 2013).

8.1.2 Entomophagy in the West

Entomophagy (the practice of eating insects) dates back to the early days of humans (Sponheimer et al. 2005) and is a current practice in over 100 countries (particularly in Latin America, Asia and Africa) with more than 2000 species being consumed (Jongema 2017). Insects can be consumed for a variety of reasons: may act as complementary nutritional sources when traditional ones are not available (Randrianandrasana and Berenbaum 2015) or don't fulfill all the nutritional requirements (Bukkens 1997), or can be consumed as delicacies, due to their sensory properties (Nonaka 2009). There is also a wide variety in the way that insects are consumed: initially, they were eaten alive (van Huis 2011), but they started to be cooked (Yen 2009) and in some countries have reached a gourmet status (Nonaka 2009).

In Europe and North America, the use of insects as food has been historically neglected, mainly due to the abundance of other protein sources that are more easily available and can guarantee a good energy intake (van Huis et al. 2013). Nonetheless, there are spontaneous cases of using insects in the food industry such as the cazu marzu (traditional goat cheese from the Sardinia region) or the food dye carmine. In the last few years, a growing interest within the academic community and the emergence of several hundred companies dedicated to the production of insects for human consumption has gradually changed this scenario. Presently, there are several commercial food products incorporating insects, like protein bars (e.g., Chapul or Exo in the USA, or Jimini's in the UK) or burgers/meatballs (e.g., Damhert in Belgium or Essento in Switzerland), though these are included in a processed and non-visible form. In the West, the most commonly used insect species as human food are mealworms, crickets, grasshoppers and locusts (van Huis 2013). While the uncertainty of their legal status (especially in the European Union) (Belluco et al. 2017) and doubts surrounding food safety—namely high microbiological loads (Vandeweyer et al. 2017) and risk of developing allergic reactions (Ribeiro et al. 2018; Ribeiro et al. 2019a)—have hindered the progress of entomophagy in the West, the greatest barrier seems to be related to the rejection of edible insects by Western consumers (Cunha and Ribeiro 2019).

8.1.3 Nutritional and Environmental Advantages of Insects' Consumption

Edible insects have a high nutritional value, mainly due to their protein content, with cricket species having 60–70% on a dry matter basis, while mealworm species have a protein content ranging from 50 to 60% on a dry matter basis. Furthermore,

the protein fraction of edible insects has great quality, having a sum of essential amino acids and good digestibility, very similar to other common protein sources. Fat content is also high (especially in some mealworm species), with insects being generally rich in polyunsaturated fatty acids and having a high ω6/ω3 ratio. In terms of micronutrients, edible insects are excellent sources of minerals (P, Fe, Zn, Cu, Mn and Se) and some vitamins (riboflavin, pantothenic acid, folic acid and biotin) (Ribeiro 2017).

Besides their nutritional value, insect rearing also presents advantages for environmental sustainability: production of insects for human consumption or feed leads to a lower emission of Greenhouse Gases and lower production of ammonia when compared to meat production (Oonincx et al. 2010). Their production also requires small areas of agricultural land per kilogram of protein produced (Oonincx and de Boer 2012) and leads to lower water use (van Huis 2013).

When compared to cattle and poultry, insects also present a more favorable feed conversion ratio, which allows for less feed to be used (van Huis 2013). Insects can also use organic waste as feed, which can also help to diminish food waste (Tabassum et al. 2016).

8.1.4 Sensory and Consumer Perspectives on Edible Insects

8.1.4.1 Entomophagy Rejection

Acceptance of entomophagy in the West is very low, with studies reporting that only 30–40% of Western consumers accept insects as food (Castro and Chambers IV 2018; Cunha et al. 2015). The main factors that control rejection of edible insects are food disgust and food neophobia (Cunha et al. 2015; Hartmann et al. 2015; Verbeke 2015), although food disgust seems to play a greater role than food neophobia (Hartmann and Siegrist 2018; La Barbera et al. 2018).

The disgust sensitivity scale (Haidt et al. 1994), modified by Olatunji et al. (2007), shows that there are three interculturally stable disgust dimensions: basic disgust provoked by the ingestion of non-edible objects or repulsive animals; disgust related to the animal nature of humans (e.g. observing a corpse); disgust based on contamination, induced by objects or situations that can contaminate an individual such as drinking from a glass utilized by someone else. Typically, disgust sensitivity lowers with age and women tend to show a greater disgust sensitivity than men (Hamerman 2016; Olatunji et al. 2007), which can explain why men have a higher acceptance of edible insects than women (Hartmann et al. 2015; Verbeke 2015). Food disgust is a primary emotion that can lead to the rejection of foods that consumers perceive as harmful (Chapman and Anderson 2012; Martins and Pliner 2006). Although there are certain predispositions to the acceptance/rejection of food (humans are born with a predisposition to accept sweet taste and reject bitter taste (Mennella and Bobowski 2015), individual food preferences are mostly dependent on the social and cultural environment (Fischler 1980, 1988). The elicitors of

disgust can also be influenced by the cultural and social environment of the individuals (Rozin and Haidt 2013), as seen in the case of insects that are regularly consumed in several regions but provoke disgust in Western consumers. Western consumers do not view insects as food, associating them with vectors of disease, pests, spoiled food, dirtiness and lack of hygiene (Cunha et al. 2014; Looy et al. 2014; Rozin et al. 1986). Furthermore, the observation of whole insects also increases disgust reactions among consumers, because it reminds them of their animal-origin (Hartmann and Siegrist 2018). Disgust towards edible insects may not necessarily reflect a deep fear of contamination/diseases and is instead driven by social and cultural norms (Deroy et al. 2015; Jensen and Lieberoth 2019).

Food neophobia is an established psychological trait that describes a person's tendency to reject or avoid eating unfamiliar foods or foods from other cultures (Pliner and Hobden 1992). It can be greatly influenced by food-disgust sensitivity (Al-Shawaf et al. 2015), although these are two different psychological constructs (Hartmann and Siegrist 2018; La Barbera et al. 2018). This rejection can be a result of unknown origins or expected harmful consequences from consumption (Martins and Pliner 2006) but can also happen due to fear of bad sensory experiences (Pelchat and Pliner 1995). This situation applies to insects because insect-based products have low expectations of liking, sensory-profiling or even emotional-profiling (Cunha and Ribeiro 2019).

8.1.4.2 Strategies to Improve Edible Insects' Acceptance

The negative effect of food neophobia on one's willingness to eat insects can be attenuated through several rational discourses. Increasing consumers' familiarity with insects, especially through tasting sessions, can have a significant positive effect on the acceptance of insects as food (Cunha et al. 2014; Hartmann et al. 2015; Sogari et al. 2019), even for the insect species that are more accepted (Fischer and Steenbekkers 2018). Highlighting the nutritional and/or environmental benefits that are associated with the consumption of insects can be another strategy. However, it will only be truly effective for consumers who are already prone to changes in their dietary habits in accordance with their nutritional and/or environmental choices (Deroy et al. 2015; Hartmann et al. 2015; Verbeke 2015). This is caused by the fact that consumers aren't willing to give up foods conveying positive experiences (sensory properties, price, availability, ability to fit in current diets) for others who only guarantee environmental, nutritional or health-related benefits (House 2016).

Nonetheless, sensory evaluation is one of the key points evaluated by consumers when making food choices (Cunha et al. 2018), and increasing the sensory appeal of edible insects can be a more effective strategy than most communicational strategies (Hamerman 2016; Myers and Pettigrew 2018). Furthermore, developing tasty insect-based products, while associating them with positive gastronomic experiences, can lead to a lower incidence of disgust (La Barbera et al. 2018). The most common strategy to improve the sensory appeal of insect-based products is to associate insects with known flavors and dishes while incorporating them in a processed,

non-visible form (Gmuer et al. 2016; Hartmann et al. 2015; Hartmann and Siegrist 2016). This is evident as both in the food industry and scientific articles most products containing insects are incorporated in a processed form such as flour, but the food matrix to be used may affect the most adequate form to incorporate the insects (Cunha and Ribeiro 2019).

Nevertheless, the sensory properties of products incorporating insects are poor and bad taste has been identified as one the major reason why consumers don't repeat the purchase of these kinds of products (House 2016). The inclusion of edible insects into food products can also lead to lower hedonic scores, less willingness to eat and poorer sensory profiles associated with negative attributes (Cunha and Ribeiro 2019; Ribeiro et al. 2019b).

Lastly, for insects to have an established place in the Western food market it is important to find a correct food categorization for them (Deroy et al. 2015) and that consumers deem the incorporation of insects as appropriate (Tan et al. 2016a, 2016b). Insects are usually presented as meat substitutes (Deroy et al. 2015) or incorporated into snack-type foods (Clarkson et al. 2018), but with these categorizations, insects are competing with already-existing food practices and are subjected to wide criteria of selection (e.g. price, sensory properties, availability, convenience) which hinders their incorporation into the regular diet of consumers (House 2016, 2018).

8.2 Goal

Considering that bread is a staple food in many Western societies, it is relevant to evaluate this food as a vector to promote the consumption of alternative protein sources, such as edible insects. In line with this thought, the main goal of this work was to assess the best predictors of acceptance of bread incorporating edible insects or edible insect protein. An online survey among regular consumers of bread was applied that sought to characterize their attitudes towards entomophagy (familiarization, motivation to try insects, willingness to consume insects, recognition of edible insect species), willingness to consume different types of bread incorporating insects and their levels of Food Neophobia and Disgust towards insects. A binary logistic regression model was applied to assess which variables better predicted the intention to consume bread incorporating insects.

8.3 Material and Methods

8.3.1 Questionnaire

An online questionnaire was applied, using Google Forms® technology, to regular bread consumers (adults and residents of mainland Portugal) between April 29th, and June 17th, 2018. A total of 282 valid answers were obtained. The questionnaire was divided into three major groups:

Group 1: Evaluation of bread consumption and purchase habits;

Group 2: Assessment of willingness to try new food products incorporating edible insects;

Group 3: Socio-demographic characterization of the participants.

8.3.1.1 Group 1

The frequency of bread consumption was evaluated based on the daily consumption of bread in the previous week. A multiple-choice increasing scale (ranging from 'none' to '4 times or more per day') was utilized. If the respondent answered "none" or "less than once a day", they would have to answer another question justifying the low consumption of bread. The answer to these two questions determined whether the respondent would be included in the sample study, as it was intended to only work with regular consumers of bread.

Additional behavioral questions included which types of bread were most consumed (white wheat bread, mix Multigrain bread, whole grain bread, etc.). The final question of this group aimed to determine if the respondents had consumed special varieties of bread in the previous year.

8.3.1.2 Group 2

Q1 - Familiarity with entomophagy was assessed through the application of the following questionnaire, adapted from Verbeke (2015), with the participant choosing the option that best describes:

No, I have never heard of the eating of insects.

I've heard that a few insects are edible.

I've heard of the eating of insects in other cultures (i.e. African and Asian).

I've heard of the eating of insects at some restaurants.

I have heard of the eating of insects but don't know what it means.

Yes, I have heard of the eating insects and I know what it means. Q2 - Willingness to try different types of bread supplemented with insects ("None"; "Whole grain bread with insect flour"; "White wheat bread with insect flour"; "Multigrain bread with dehydrated insects"; "Fiber bread with insect protein powder"; "Other") was assessed with a multiple-choice questionnaire.

Q3 - The potential for the inclusion of edible insects in the diet, adapted from Verbeke (2015), was assessed with the choice of one of the following statements: "As a meat protein substitute", "As a new ingredient to add" and "I don't actively eat insects".

Q4 - The motivation to experiment edible insects was evaluated with a multiple-choice tick all that apply questionnaire ("Nothing"; "Taste"; "To experiment new products"; "Curiosity"; "High nutritional value"; "Sustainability"; "Other").

Q5 - The Reduced Food Neophobia Scale (Pliner and Hobden 1992), modified by Ritchey et al. (2003)) was used to assess respondents' neophobia, using a 7-level

ordinal scale anchored to the extremes, with the level 1 "strongly disagree" and level 7 "strongly agree" incorporating two items about food neophobia and two items about food neophilia.

In the same question, the level of insect repulsion was evaluated using the Disgust towards insects scale ((Cunha et al. 2015), adapted from Rozin et al. (2014)), evaluated over a 7-point anchored scale, going from 1-" Strongly disagree", to 7-" Strongly agree":

The idea of insects makes me nauseous.

The idea of insects makes me ill.

Eating insects is disgusting.

I am offended by the idea of eating insects.

If an insect crawls on my favorite food I won't eat it.

Q6 - The knowledge of edible insects was also assessed through an open question: "Mention, if you know, up to four insects you consider edible."

8.3.1.3 Socio-Demographic Characteristics

Information was collected about the socio-demographic characteristics of the respondents: gender; age; civil status; education level; professional status; net monthly family income and district of residence.

8.3.2 Sampling

A non-random sampling was structured by age and education level. Respondents were distinguished in three major age groups: 18 to 34 years old; 35 to 54 years old and over 55 years old and according to their level of education (with and without higher education), obtaining a total of six groups, to verify the effect of the age and educational level in the variables under study, thus maximizing the information.

8.3.3 Statistical Analysis

Statistical analysis of the data related to the questionnaires was performed through Statistical Package for the Social Sciences - SPSS® for Windows, version 25.

An exploratory factorial analysis was carried out to determine the applicability of the Disgust towards insects' scale and the Food Neophobia subscales, as variables that predict the acceptance of insect consumption among regular consumers of bread. For each scale, the applicability of the factorial analysis was assessed through the Kaiser-Mayer-Olkin (KMO) coefficient and the internal consistency with the α-Cronbach.

This analysis aimed to predict the acceptance of bread supplemented with insects among regular bread consumers using a binary logistic regression model (Hosmer and Lemeshow 2000). Through this model, we tried to predict the acceptance of the consumption of bread incorporating insects (assessed through the dichotomization of the Q2-Group 2 with 0 corresponding to "never" and 1 corresponding to any other answers). The model expresses the variation in the probability of acceptance of the consumption of bread supplemented with insects according to the following expression:

$$p_i = \left(\frac{e^{(Z_i)}}{1 + e^{(Z_i)}} \right) = \left(\frac{1}{1 + e^{-(\beta_0 + \beta_1 X_{1i} + \ldots + \beta_k X_{kn})}} \right) \tag{8.1}$$

It was intended to determine the relationships between the variables and predict the value of the variable dependent (or response) from a set of independent variables (or predictors). These relationships may be of functional dependence (the magnitude of the dependent variable is a function of the magnitude of the independent variable(s), although the reverse is not applicable) or mere association (none of the variables can be dependent on the other, varying only together) (Marôco 2010). To facilitate the interpretation of the model, variables were dichotomized, such as the education level - higher education (0-no; 1-yes); the intention to consume insects (0- "nothing"; 1-yes), familiarization with insect consumption (0- "I had never heard of eating insects"; 1- "I heard…"), or the consumption of special varieties of bread (0-no; 1-yes).

Starting from a saturated model, with the various variables under analysis, the best model was selected through stepwise backward elimination, based on Ward statistics. The quality of the final model was evaluated, through its correction on the prediction and the pseudo coefficient determination (R^2) of Nagelkerke.

8.4 Results and Discussion

8.4.1 Socio-Demographic Characteristics of the Participants

Initially, 282 questionnaires were obtained. After analysis of the answers to the frequency of consumption of bread, questionnaires from participant whom either "do not consume bread" or consumed bread "less than once per day" were eliminated, given that they did not represent a population of regular consumers of bread. This way, 226 valid questionnaires were obtained for this study.

Concerning the socio-demographic characteristics of the participants (Table 8.1), 61% of the participants were female and 65% had age between 35 and 54 years old. The age of the participants ranged between 22 and 78 years old (average 42.0 ± 11.3). Concerning the education level, most of the participants (74.8%) had high education. Furthermore, most of the participants were married (68.1%). Regarding the

Table 8.1 Socio-demographic characteristics of the participants

Characteristic		*n* (%)
Sex	Male	88 (38.9%)
	Female	138 (61.1%)
Age group (years)	[18;34]	51 (22.6%)
	[35;54]	147 (65.0%)
	≥55	28 (12.4%)
	Average (±SD)	42.0 (± 11.3)
Civil status	Single	53 (23.5%)
	Married	154 (68.1%)
	Divorced/separated	17 (7.5%)
	Widower	2 (0.9%)
Education level	Without higher education	57 (25.2%)
	With higher education	169 (74.8%)
Net monthly family income (€/month)[a]	[485; 900]	23 (10.2%)
	[900; 1500]	50 (22.1%)
	[1500; 2400]	66 (29.2%)
	[2400; 3600]	40 (17.7%)
	≥3600	11 (4.9%)
	Don't know/did not answer	36 (15.9%)
Region of residence	North	97 (42.9%)
	Center and south	129 (57.1%)

[a]National minimum wage of 580 € /month

monthly household income, most of the participants had values between 1500 and 2400 €/month (29.2%).

8.4.2 Characterization of Bread Consumption

Regarding the types of bread that are consumed regularly, there is a predominance of mixture bread (52.0%) and white wheat bread (49.3%), followed by multigrain bread (38.1%), rye bread (28.7%) and whole grain bread (23.3%).

Most participants did not consume any type of specialty bread (65.9%) in the last year (Table 8.2). Of the different types of specialty bread, the ones which were most consumed were low salt bread (14.1%), fiber+ bread (12.3%) and prokorn bread (12.3%).

8.4.3 Familiarization with Insect Consumption

Regarding the level of familiarization with insect consumption (Table 8.3), only 10.2% of the participants did not know this practice. Most of the respondents knew that insects are eaten in African and Asian cultures (38.9%) or that insects are eaten,

understanding what that means (35.8%). This high degree of familiarization with the concept of entomophagy among Portuguese consumers has already been reported (Cunha et al. 2015), and has very similar levels to studies with other European (Verbeke 2015) and North American (Tao and Li 2018) consumers. Familiarization with the concept of entomophagy can increase its acceptance (Cunha et al. 2014; Hartmann et al. 2015), but performing tasting sessions can have a greater effect on acceptance since consumers become more familiarized with the sensory properties of insects (Sogari et al. 2017, 2019).

8.4.4 Willingness to Try Edible Insects

Most of the respondents (58%) would not regularly consume insects in their diets, a very similar percentage to the participants who would not try any type of bread incorporating insects. On the other hand, 23.9% of them would use them as new ingredients and 15.5% would use insects as meat substitutes.

The majority of the participants (57.8%) would not consume any of the types of bread supplemented with insects (Table 8.4). Other studies with Western consumers have shown similar levels of unwilling tasters of products incorporating processed insects (Castro and Chambers Iv 2018; Kostecka et al. 2017; Lammers et al. 2019).

Concerning the different types of bread supplemented with insects, "special" types of bread (whole wheat and fiber) incorporating processed insects had the highest acceptance (26.2% and 25.8%, respectively), being higher than the acceptance of white wheat bread with insect flour (16.0%). The type of bread with the lowest acceptance was Multigrain bread with dehydrated insects (13.3%). These results mirror the higher acceptance of products incorporating insects in a processed, non-visible form that is extensively reported in the literature (Gmuer et al. 2016; Hartmann et al. 2015; Hartmann and Siegrist 2016). The higher preference for specialty bread (whole-grain bread and fiber bread) over white wheat bread could have happened because consumers of specialty bread are more predisposed to consume bread incorporating insects (Table 8.6).

Table 8.2 Types of specialty bread consumed within the last year

Type of specialty bread	*n* (%)
Don't consume these types of bread	145 (65.9%)
Low salt bread	31 (14.1%)
Fiber+ bread	27 (12.3%)
Prokorn bread	27 (12.3%)
"São Coração" bread	14 (6.4%)
Gluten-free bread	12 (5.5%)
"Vida" bread	9 (4.1%)
"São Diabéticos" bread	2 (0.9%)

Table 8.3 Level of familiarization with insect consumption

Statement	*n* (%)
I have heard that in some African and Asian cultures insects are eaten	88 (38.9%)
I have heard that insects are eaten, and I know what that means	81 (35.8%)
I have heard that some insects are edible	28 (12.4%)
I have never heard about eating insects	23 (10.2%)
I have heard that in some restaurants, insects are eaten	4 (1.8%)
I have heard that insects are eaten, but I don't know what that means	2 (0.9%)

Table 8.4 Willingness to eat different types of bread incorporating insects

Types of bread	*n* (%)
None	130 (57.8%)
Whole grain bread with insect flour	59 (26.2%)
Fiber bread with powdered insects	58 (25.8%)
White wheat bread with insect flour	36 (16.0%)
Multigrain bread with dehydrated insects	30 (13.3%)
Other	6 (2.7%)

8.4.5 Motivations to Try Insects

Most respondents (40.4%) demonstrated a great reluctance to eat insects, mentioning that nothing would lead them to try them. Curiosity or willingness to try new products (53.8%) is the factor most respondents mentioned that would motivate them to try insects. The sustainability (30.2%) and high nutritional value (20.4%) are also important factors that would motivate participants to try edible insects. Previous works have reported that consumers who are more willing to try edible insects are looking for new food experiences and/or are aware of the nutritional and environmental impacts of their food choices (House 2016; Sogari et al. 2017). Lastly, only 8.0% of the participants mentioned the taste of insects as motivating factors to try edible insects, which further highlights that either consumers have poor knowledge regarding the sensory properties of insects or have expectations of bad sensory experiences caused by consumption of insects (Cunha and Ribeiro 2019).

8.4.6 Edible Insect Species

Regarding the insect species that the respondents considered edible (Table 8.5), the most mentioned species were grasshoppers (30.5%), crickets (18.8%), mealworm (16.7%) and ants (15.6%). The presence of grasshoppers, crickets and mealworms

Table 8.5 Species of insects' participants deemed as edible

Edible insect species	*n* (%)
Grasshoppers	86 (30.5%)
Crickets	53 (18.8%)
Mealworm	47 (16.7%)
Ants	44 (15.6%)
Cockroaches	23 (8.2%)
Scarab	12 (4.3%)
Spiders and scorpions	12 (4.3%)
Worms	10 (3.5%)
Caterpillars	6 (2.1%)
Cicadas	4 (1.4%)
Others	12 (4.3%)

is not surprising since these species are currently the most marketed in the West and the ones which are more accepted by consume**rs (**Fischer and Steenbekkers 2018**).**

8.4.7 Disgust and Food Neophobia

The "Disgust towards insects" scale has a robust factorial structure (Table 8.6), with all the items of the scale presenting a high factorial loading. The reliability of this scale was also good (α-Cronbach = 0.8**84).**

On the other hand, the Reduced Food Neophobia Scale proved not to be unidimensional, splitting into two subscales: Food Neophilia and Food Neophobia.

Furthermore, the Food Neophilia subscale and the Disgust Scale had a negative correlation (−0.172) as well as the Food Neophilia and Food Neophobia subscales (−0.268) (Table 8.7). On the other hand, the Food Neophobia subscale had a strong correlation with the Disgust Scale (0.553).

8.4.8 Variables Prediction of Acceptance of Insects

Results obtained from the application of the binary logistic regression model (Table 8.8), following a stepwise approach, allowed to observe which variables significantly predict the acceptance of bread supplemented with insects. The main predictor of the acceptance of the bread was the willingness to try insects, given that participants who are willing to eat insects have a probability of 40.6 times higher to accept consuming bread with insects than those who are not willing to try insects.

Gender also plays a significant role, with men being 2.68 times more likely to accept bread with special types of insects. Several studies have shown that males have a higher acceptance of entomophagy (Hartmann et al. 2015; Verbeke 2015;

Table 8.6 Factorial analysis of the Disgust towards insects' scale and of the food neophilia and food neophobia subscales

Scale/Items	Average (±S.D)	Loadings
Disgust towards insects scale (KMO = 0.844; explained variance = 68.5%; α-Cronbach = 0.884)	3.7 ± (0.12)	
Just thinking about insects makes me nauseous	3.6 ± (0.15)	0.892
Just thinking about insects makes me sick	3.3 ± (0.16)	0.874
Eating insects is disgusting	4.1 ± (0.15)	0.867
I get offended by the idea of eating insects	2.5 ± (0.13)	0.789
If an insect crawls over my favorite food I no longer eat it	4.9 ± (0.14)	0.702
Food neophilia (explained variance = 39.4%; α-Cronbach = 0.730)	4.3 ± (0.12)	
I like food from different countries	4.7 ± (0.14)	0.769
I constantly try new and different foods	4.0 ± (0.13)	0.729
Food neophobia (explained variance = 36.0%; α-Cronbach = 0.603)	3.30 ± (0.11)	
If an insect crawls over my favorite food I no longer eat it	2.6 ± (0.12)	0.587
If I do not know the ingredients in a food, I do not try it	3.4 ± (0.13)	0.548

Table 8.7 Correlation between the disgust scale and the Food Neophobia subscales

Scale/Scale	Disgust scale	Food neophilia
Food neophilia	−0.172	
Food neophobia	0.553	−0.268

Woolf et al. 2019). This can be explained by the fact that in general, men are less sensitive to disgust than women and have a lower animal reminder disgust sensitivity (Hamerman 2016).

Consumption of special varieties of bread can also positively influence the acceptance of bread with insects (Exp(β) = 2.54). Consumers of these types of bread have been described as health-conscious in their food choices (Meyerding et al. 2018), which can lead to a higher acceptance of novel ingredients such as insects.

On the other hand, each point increment on the neophilia scale lead to an increase (Exp(β) = 1.56) in the acceptance of bread incorporating insects. This data is consistent with the identification of 'curiosity' and 'willingness to try new products' as the main motivators to try edible insects. Conversely, a point increase in the Disgust Scale halved the probability of accepting bread with insects (Exp(β) = 0.51). Previously, both Food Disgust and Food Neophobia have been identified as mains factors controlling acceptance of insects as food (Cunha et al. 2015; Hartmann et al. 2015; Verbeke 2015), though recent studies (Hartmann and Siegrist 2018; La Barbera et al. 2018), as well as the results of our work, have shown that Food Disgust plays a greater role in predicting willingness to try edible insects, further highlighting that the consumption of insects needs to be associated with positive experiences in order to reverse the induction of dis**gust.**

The R^2-Nagelkerke of 0.676 indicates a high predictability of this model. This model predicts the answer "No availability to consume bread with insect protein" in

Table 8.8 Variables prediction of acceptance of bread incorporating insects

Significant variables	B	Sig	Exp(B)
Willingness to try insects (0 = no; 1 = yes)	3.705	<0.001	40.6
Sex (0 = female; 1 = male	0.984	0.023	2.68
Consumption of specialty breads (0 = no; 1 = yes	0.933	0.032	2.54
Food Neophilia subscale	0447	0.001	1.56
Disgust scale	−0.678	<0.001	0.51
Constant	1.088	0.139	2.97

R^2-Nagelkerke = 0.676

85.4% of the cases and the answer "Some availability of consuming bread with insect protein" in 83.3% of the cases. Globally, this equation/model, predicts 84.5% of the answers. Moreover, the set of variables allows to obtain a model of prediction with a sensitivity value of 80.8% (80/99) and specificity of 87.4% (111/127).

8.5 Conclusion

The results of this work support the current knowledge regarding the Western consumer's attitudes towards entomophagy. The participants of this study showed a high degree of familiarity with the concept of entomophagy, which could have also influenced the insect species they deemed as edible. Concerning the willingness to try different types of bread incorporating insects, a higher acceptance was verified for special-type bread (e.g. wholegrain and fiber bread) incorporating insects in a processed, non-visible form. Consumers of special-type bread were also shown to have higher acceptability of edible insects, most likely because these consumers are more willing to try new ingredients and are more conscious of the health and nutritional effects of their food choices.

Disgust towards insects plays a major role in the rejection of entomophagy, which could have contributed to the gender impact on the acceptance of insects. The role of food disgust was greater than food neophobia, although the subscale of Food Neophilia also predicted the acceptance of edible insects, which is not surprising since the novelty of edible insects is one the main factors that can lead to consumers tasting them.

These results further confirm the necessity of continuing to popularize the concept of entomophagy, so consumers become more familiarized which contributes to higher acceptance. Furthermore, it is also necessary to promote the advantages associated with the consumption of insects to reach the types of consumers who are more predisposed to include insects into their diets. Lastly, positive ideas have to be associated with the consumption of insects to reduce the reactions of disgust triggered by insect consumption.

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Chapter 9
Potential Use of Aqueous Extracts of Kombu Seaweed in Cream Cracker Formulation

Joaquina Pinheiro, Elsa M. Gonçalves, and Rui Ganhão

9.1 Introduction

Edible seaweeds have been defined as healthy food, mainly due to their richness on minerals and dietary fibers, especially the Kombu (*Laminaria japonica*), Wakame (*Undaria pinnatifida*), tengusa (*Gelidium crinale*) and mozuku (*Cladosiphon okamuranus*) that have been highly and traditionally consumed in Japan (Shirosaki and Koyana 2011; Pereira 2016). In Europe, some countries like Portugal, France, Belgium and Italy have authorized a few species of seaweed that can be used in the food supplements and in this case the products are presented in capsules/tablets with seaweed powder or extracts or in pure seaweed powder. Also, the purchase of edible seaweed can be done in a local market of natural food products since they are related and connected to consumer well-being.

The brown seaweed Kombu (*Laminaria japonica*) is a nutritious and edible food, rich in biologically compounds such as amino acids, polyunsaturated fatty acid, alginate, vitamins, and micronutrients like K, Na, Ca, Mg and others (Kim and Bhatnagar 2011; Pereira 2016). The content of minerals of this particular seaweed are water-soluble salts of potassium and sodium (chlorides and sulfates) (Kim and Bhatnagar 2011) which contributes to apply this seaweed as a substitute of salt.

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In Portugal, the Portuguese's diet is associated with excessive salt (or sodium Na) consumption and according to the Ministry of Health this action leading to health problems such as high blood pressure and certain cancers such as stomach (EIPAS—Estratégia Integrada Para A Promoção Da Alimentação Saudável 2018). In this sense, last year, an agreement was signed between the Food Industry and Ministry of Health, to find solutions to this problem, reducing the salt amount of processed foods such as cookies, bread, French fries among others products and prevent the risk of diseases (Goiana-da-Silva et al. 2019). However, this reduction could provide critical technological functions, which can impact dough processability and product quality attributes, including sensorial and shelf-life functions. Therefore, the replacement salt strategy should provide similar technological functions without negatively impacting dough processing and cookie qualities.

Cookies are by definition, a convenient food, ready-to-eat, cheap with an important position in the snack food category and highly consumed by people of all age groups in the world (Batista et al. 2017). Cookies have been reported as rich in carbohydrates and fats with good sources of proteins and minerals (Fradinho et al. 2015).

To our knowledge, no available reports regarding the evaluation of adding the aqueous extracts of Kombu seaweed on cream cracker formulation were done. This study reports a promising way to extract consumer health-benefits compounds from a natural resource by conventional heat treatment. The main objectives of this research were to evaluate the potential of using the aqueous extracts of Kombu seaweed at different concentration level, as natural ingredients into cream cracker formulation. Moreover, the behavior of all biscuit's samples regarding the determinants of quality attributes for the consumer's point-of-view, like color, texture, total phenolic content and antioxidant capacity, was assessed during 35 days of storage at room temperature.

9.2 Materials and Methods

9.2.1 Raw Materials

The dried Kombu seaweed (*Laminaria Japonica*) and the ingredients for the preparation of cream cracker, like dehydrated organic yeast, flour, salt, sodium bicarbonate, Parmesan cheese and oregano were purchased at *Caldas da Rainha* (Portugal), in a natural food market.

The dried Kombu seaweed has an interesting nutritional composition presenting the major proportions in fiber and protein content as can be observed in Table 9.1.

Table 9.1 Nutritional composition of dried Kombu seaweed used in this study

Component	(g.100 g^{-1})
Protein	9
Lipids	1.8
Carbohydrates	13.1
Fiber	36
Salt	8.6
Energy	176 kcal

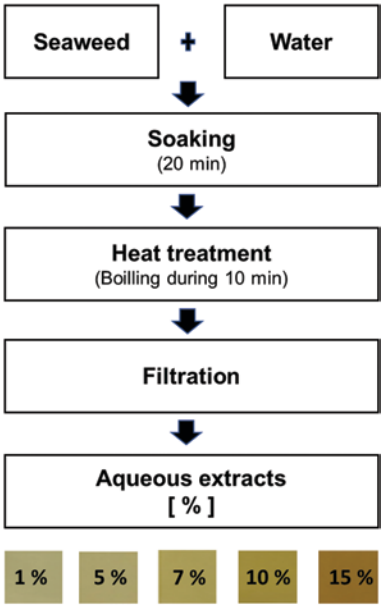

Fig. 9.1 Sequence of aqueous extract of Kombu seaweed preparation

9.2.2 Methods

9.2.2.1 Preparation of Cream Cracker with and without Aqueous Extracts of Kombu Seaweed

The aqueous extracts of kombu seaweed, at five concentration levels: 1%, 5%, 7%, 10% and 15% was prepared according to the modified method by Ak and Turker (2018) as observed in Fig. 9.1. Briefly, the seaweed was weighted, according to the concentration level of aqueous extract (1%, 5%, 7%, 10% and 15%), then soaked with water for 20 min and boiled for 15 min. After, the mix was cooled, filtered to remove the undesirable parts of seaweed fragments and stored at 5 °C to prevent changes.

For cream cracker preparation a typical recipe was followed, by weighted 10 g of dehydrated organic yeast, 160 mL of warm water (according to the concentration level of seaweed: 1%, 5%, 7%, 10%, 15%), 200 g of flour, 1 g of salt, 0.5 g of sodium bicarbonate, 2 g Parmesan cheese and oregano as presented at Fig. 9.2. Two

control biscuits samples are considered, with and without salt, identified as CTR1, and CTR2, respectively. After mixing and fermented, the mass of biscuit was cut into a typical cream cracker biscuit shape and baked to 200 °C for 10 min in an oven preheated. After cooled, all the cream cracker samples were packed in vacuum, protected from light, and stored at room temperature for quality evaluated at 0, 7, 15, 21, 27, 35 days of storage.

9.2.2.2 Characterization of Aqueous Extracts of Kombu Seaweed

After the preparation of each concentration level of aqueous extracts of Kombu seaweed, a mixture using the ethanol as solvent, at a ratio of 1/10 (v/v) was performed. After centrifugation (Centrifuge, Eppendorf, 5810R Madrid, Spain) of the mixture, the supernatant was collected and used as an extract sample of Kombu seaweed for antioxidant capacity and total phenolic content.

The antioxidant capacity was realized by DPPH radical scavenging activity according to the methodology described by Brand-Williams et al. (1995). Briefly, 50 μL of extract sample was reacted with 150 μL of DPPH solution (150 μmol.L^{-1}) during 1 h at room temperature in the dark. Then, the reaction was measured at 517 nm in a microplate reader (Synergy H1 Multi-Mode Microplate Reader, BioTek® Instruments, Winooski, VT, USA). The sample blank was only the solvent with no sample. The percentage of inhibition was expressed as radical scavenging

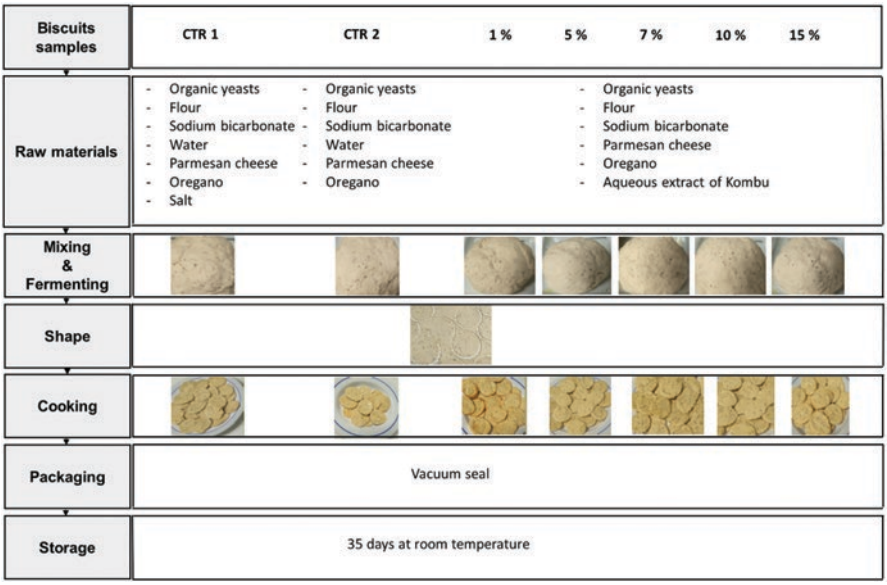

Fig. 9.2 Flow-chart of cream cracker without (CTR1- with salt; CTR2-without salt) and with aqueous extracts of Kombu seaweed (1%, 5%, 7%, 10% and 15%) preparation

activity (% RSA) as observed in Eq. (9.1) and resulted in three measurements per each concentration level of aqueous extracts of Kombu seaweed.

$$\%\text{RSA} = \frac{\left(\text{Abs}_{\text{blank}} - \text{Abs}_{\text{sample}}\right)}{\text{Abs}_{\text{blank}}} \times 100 \tag{9.1}$$

where, the $\text{Abs}_{\text{blank}}$ and $\text{Abs}_{\text{sample}}$ are the absorbance of DPPH solution and the mixture of DPPH solution and sample, respectively.

Total phenolic content (TPC) was determined by Folin-Ciocalteu method (Singleton and Rossi 1965) and adapted for 96-well plate assay. Briefly, 20 μL of extract sample/standard was mixed with 100 μL of Folin-Ciocalteau reagent (1/10; v/v)) and after 4 min, 80 μL of Na_2CO_3 solution (7.5%, m/v) was added. The reaction occurs at room temperature for 2 h and the absorbance of the mixture was measured at 750 nm using a microplate reader (Synergy H1 Multi-Mode Microplate Reader, BioTek® Instruments, Winooski, VT, USA). The sample blank was performed using the solvent with no sample. Gallic acid (0.05–0.25 mg.mL^{-1}) was used for the standard calibration curve. TPC was expressed as milligrams of gallic acid equivalents per 100 grams of seaweed (mg GAE.100 g^{-1}) and resulted in three measurements per each concentration level of aqueous extracts of Kombu seaweed.

9.2.2.3 Evaluation of Physical Quality Attributes of Cream Cracker

The **diameter** and **thickness** of cream cracker samples (control and enriched with aqueous extracts of Kombu seaweed) were performed using a caliber (King-Tools, SP, Brazil) and a ten-measurement average was performed in each group sample of cream cracker.

Color measurement was performed in a colorimeter (Minolta chroma Meter, CR-300, Osaka, Japan) previously calibrated with a standard white tile (L^* = 97.10, a^* = 0.19, b^* = 1.95). The color coordinates of CIE $L^*a^*b^*$ was determined using the D65 illuminant and 2° observer where the L* value represent the luminosity of samples (0—black to 100—white), a* and b* values indicate the variation of greenness to redness (−60 to +60) and blueness to yellowness (−60 to +60), respectively. Also, the tonality (hue, °h) and total difference color (TDC) (Drlange 1994), were obtained as observed in Eqs. (9.2) and (9.3), respectively:

$$^{\circ}h = \tan\left(\frac{b^*}{a^*}\right) \tag{9.2}$$

$$\text{TCD} = \sqrt{\left(L^* - L_0^*\right)^2 + \left(a^* - a_0^*\right)^2 + \left(b^* - b_0^*\right)^2} \tag{9.3}$$

Ten measurements were carried out in each sample group of cream cracker biscuits per analysis days.

The texture of cream cracker group samples was measured using a Texture Analyzer (TA.HDi, Stable Microsystem Ltd., Godalming, UK), through a penetration test using a stainless steel cylinder probe with a 2 mm diameter. The penetration test was performed at 1 mm.s^{-1} of velocity and 3 mm of penetration distance. Force-distance curves were recorded and firmness (maximum peak force (N)) from the average of eight measurements of each cream cracker group sample was performed and used as an indicator of the texture parameter.

9.2.2.4 Evaluation of Antioxidant Capacity and Total Phenolic Content of Cream Cracker

Preparation of extracts of cream cracker samples for DPPH scavenging activity and total phenolic content.

The extraction of the interesting compounds of cream cracker was performed by Roleda et al. (2019) with some modification. Briefly, a solid to liquid ratio of 1/10 (m/v) using the ethanol as solvent, was performed. The mixture was incubated in the dark at refrigerated temperature for 24 h. Then the mixture was centrifuged (Centrifuge, Eppendorf, 5810R Madrid, Spain) and the supernatant was collected and used as an extract sample.

The antioxidant capacity and total phenolic content of cream cracker samples were realized as previously described in Sect. 9.2.4.1.

9.2.3 Data Analysis

Data were subjected to analysis of variance using the Statistica v.7.0 (StatSoft Inc 2007) with a significant level of $p < 0.05$ by Tukey Honestly Significant Difference (HSD) test to determine the effects of the different concentration level of aqueous extracts of Kombu seaweed in physical quality attributes (color and texture), antioxidant capacity (DPPH radical scavenging activity) and total phenolics content of cream cracker.

9.3 Results

9.3.1 Characterization of Aqueous Extracts of Kombu Seaweed

The color of aqueous extracts of Kombu seaweed, after preparation, can be observed in Fig. 9.3.

By observation of color obtained in aqueous extracts of Kombu seaweed, an increase of intensity was revealed when the concentration levels augmented from

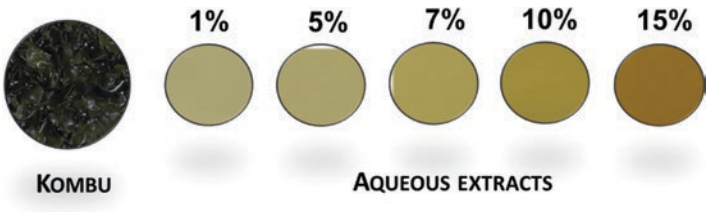

Fig. 9.3 Illustration of aqueous extracts of kombu seaweed color

1% to 15% after preparation. The combination of water, as solvent and heat as extraction methodology, seems to provide the solubilization of promising compounds that can be affecting the global appearance of biscuits and improves the consumer's health, for instance, the carbohydrates content.

As reported by Pereira (2016), the brown seaweed contains the carbohydrate mannitol, that can be released when the seaweed is soaked in hot water. In this sense, the significant amount of mannitol that has been released can be used as a propose of sweet taste without adding calories to the food (Rioux et al. 2017). This compound varies according to environmental conditions and with external salinity as reported by Wright and Reed (1985).

The aqueous extracts of Kombu, at concentration level from 1% to 15%, exhibited a promising content of antioxidant capacity and total phenolic, as can be observed in Figs. 9.4 and 9.5.

The antioxidant capacity expressed as DPPH radical scavenging activity ranged from 41.67% to 51.79 in lowest (1%) and highest (15%) concentration of aqueous Kombu seaweed, respectively. As previously reported by Kim and Bhatnagar (2011), the antioxidant activity of *Laminaria japonica* (Kombu) is related to the efficiency and amount of fucoidan. It seems that fucoidan from *L. japonica* shows a better scavenging effect on superoxide radicals, on contrary to observe on hydroxyl radical and DPPH. Identical effect on inhibition of DPPH radical between the concentrations of aqueous extracts of Kombu seaweed can be a result of this weak efficacy of antioxidant capacity expressed by DPPH radical scavenging activity from fucoidan of brown seaweed. Only at 10% of aqueous extract concentration of Kombu seaweed, exhibited a significant effect on antioxidant capacity when compared to the lowest concentrations, 1% 5% and 7%.

The highest value of total phenolics content (4.71 mg GAE.100 g^{-1}) was obtained at a concentration level of 15% of aqueous extracts of Kombu seaweed. Our results indicate solubilization of phenolic compounds when the seaweed in water was exposed to high temperature, which favored the availability and proves the efficacy of bioactive compounds extraction from brown seaweed.

The study reported by Cernadas et al. (2019) and López-Hortas et al. (2018), showed identical effect after treated two species of brown seaweed, *Himanthalia elongate* and *Laminaria ochroleuca*, by using an eco-friendly technology.

Dependency between the concentration level of aqueous extracts and antioxidant capacity expressed by DPPH scavenging activity and total phenolic content is in

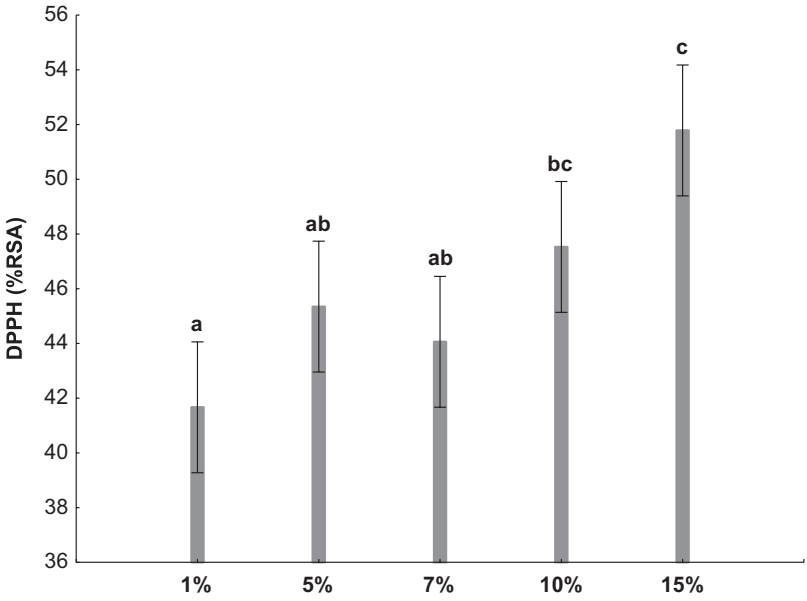

Fig. 9.4 Antioxidant capacity expressed by DPPH radical scavenging activity (% RSA) of aqueous extracts of kombu seaweed. Vertical lines denote 95% confidence intervals. Different letters indicate significant differences at $p < 0.05$ (Tukey's test) between aqueous extracts of Kombu seaweed

agreement with found by Marinho et al. (2019) where an increase of seaweed extracts concentrations lead to augment of antioxidant capacity. Moreover, the study developed by López-Hortas et al. (2019) exhibited a positive correlation between total phenolic content and DPPH capacity in *Undaria pinnatifida* suggesting that antioxidant capacity of different aqueous extracts of kombu seaweed was related to polyphenolic compounds of this kind of seaweed, such as essential oils and fucoxanthin (Wang et al. 2018; López-Hortas et al. 2019).

9.3.2 Effects of Aqueous Extracts of Kombu Seaweed in Physical Quality Attributes of Cream Cracker

In the present study, aqueous extracts of Kombu seaweed have been added into cream cracker formulation and the effects on quality attributes of color, texture and health benefits expressed by total phenolics content and antioxidant activity, was evaluated. The increase of the concentration level of Kombu seaweed improves the visual appearance of cream crackers, demonstrating a pleasant and characteristic tonality from yellow dark due to added seaweed. The diameter and thickness of all

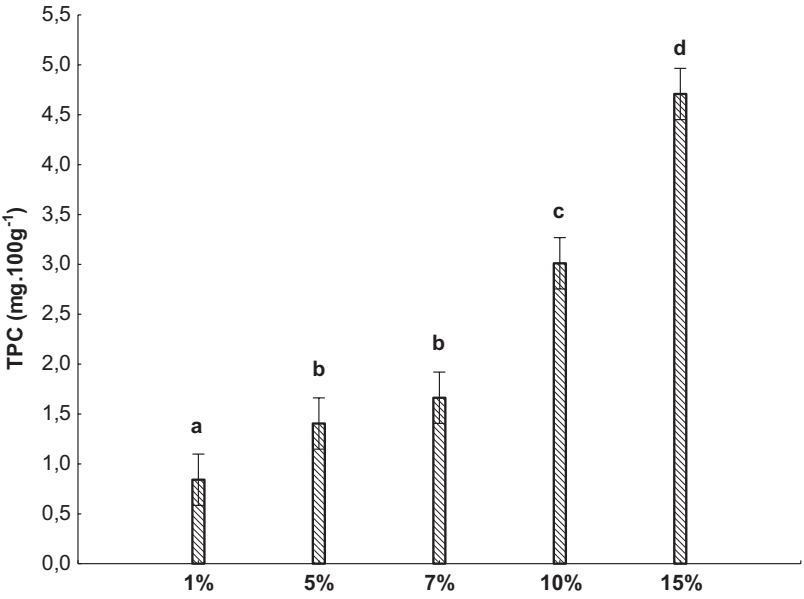

Fig. 9.5 Total phenolic content (mg GAE.100 g^{-1}) of aqueous extracts of kombu seaweed. Vertical lines denote 95% confidence intervals. Different letters indicate significant differences at $p < 0.05$ (Tukey's test) between aqueous extracts of Kombu seaweed

cream crackers were similar, showed an average value of 25.6 ± 0.45 mm and 4.1 ± 0.2 mm, respectively.

The results of color parameters (L^*, a^*, b^*, °h and TCD) of cream cracker immediately after preparation are summarized in Table 9.2**.**

As observed in other types of biscuits, the color of cream crackers depends on the baking process influencing the surface color and the expected firmness texture of products (Manley 2011).

Comparing the luminosity of both control samples, a significant difference ($p < 0.05$) was observed being the biscuit sample without salt, the significant highest one (73.98 ± 2.39, $p < 0.05$), however visually, this difference was not noticed. The addition of brown seaweed aqueous extracts decreased the luminosity in all biscuit samples leading to slight darkness on enriched biscuits samples. These luminosity differences could be provoked by the high temperature of the baking and caramelization process (Mahloko et al. 2019).

The high a^* value observed on enriched biscuits samples B1; B5 and B10 denote a favorable redness. On the other hand, regarding the highest enriched biscuit, B15, a decrease in the b^* value, was noticed. Regarding the tonality value, obtained in all biscuit samples, a range between 82 and 89 representing the red, orange and yellow color. According to the classification of color difference defined by Drlange (1994), all the cream cracker biscuits showed a clear difference compared to standard/control biscuits, with a remarkable two group: higher difference (6.0–12.0) and very

Table 9.2 Characterization of color parameters (L^*, a^*, b^*, °h, TCD) of cream cracker samples (control and enriched with aqueous extracts of Kombu seaweed)

Sample	L^*	a^*	b^*	°h	TCD
CTR1	69.54 ± 1.85^{ac}	1.85 ± 0.84^{a}	29.77 ± 3.02^{ab}	86.56 ± 1.28^{a}	0.00 ± 0.00^{d}
CTR2	73.98 ± 2.39^{d}	1.62 ± 0.55^{a}	30.93 ± 1.40^{b}	87.02 ± 0.96^{a}	5.69 ± 2.20^{b}
B1	64.34 ± 2.25^{b}	3.97 ± 1.36^{d}	33.06 ± 3.41^{d}	83.34 ± 1.91^{c}	9.00 ± 3.05^{c}
B5	63.56 ± 2.66^{b}	4.92 ± 1.06^{e}	33.84 ± 1.74^{d}	81.79 ± 1.45^{b}	8.55 ± 3.05^{c}
B7	70.54 ± 2.88^{a}	1.64 ± 0.94^{a}	29.05 ± 2.10^{a}	86.85 ± 1.56^{a}	3.98 ± 2.44^{a}
B10	67.59 ± 3.66^{c}	2.66 ± 0.61^{c}	29.68 ± 1.63^{ab}	84.89 ± 1.16^{d}	5.34 ± 2.94^{ab}
B15	70.44 ± 2.17^{a}	0.66 ± 0.41^{b}	27.77 ± 0.90^{c}	88.65 ± 0.85^{e}	4.91 ± 2.50^{a}

CTR1 standard formulation of cream cracker, *CTR2* formulation of cream cracker without salt, *B1* formulation of cream cracker enriched with 1% of aqueous extract of Kombu seaweed, *B5* formulation of cream cracker enriched with 5% of aqueous extract of Kombu seaweed, *B7* formulation of cream cracker enriched with 7% of aqueous extract of Kombu seaweed, *B10* formulation of cream cracker enriched with 10% of aqueous extract of Kombu seaweed, *B15* formulation of cream cracker enriched with 15% of aqueous extract of Kombu seaweed

TCD – the total color difference was determined, being the "control sample", CTR1 sample – the typical formulation of cream cracker (with all ingredients and without aqueous extract of Kombu seaweed)

[a-d]In the same column, different letters indicate significant differences at $p < 0.05$ (Tukey's test) between group samples of cream crackers

distinct difference (3.0–6.0). The biscuit samples identified as B1 and B5 was included in the first group and the others one (B7, B10 and B15) in the second group.

The higher color difference in all cream cracker could be due to the ingredient formulation and yellow-brown pigmentation resulting for the color of studied seaweed and from the Maillard reaction or non-enzymatic browning that depends on reducing sugars, amino acids or proteins on the biscuits surface and temperature and time baking (Pereira et al. 2013).

During storage, all cream cracker group samples evidenced the highest color difference compared to color observed on the first day (Fig. 9.6). The enriched biscuit samples with 7%, 10% and 15% demonstrated a color preservation/stabilization compared to differences encountered in the other group samples.

This color difference could be expected regarding the high level of yellow-brown pigment of brown seaweed aqueous extracts, that have a brown characteristic color of the seaweed Kombu.

The influence of aqueous extracts of Kombu seaweed on cream cracker firmness is shown in Fig. 9.7.

A significant ($p < 0.05$) difference regarding the maximum force in all cream cracker enriched with seaweed, was observed, being more pronounced with augment of the concentration level of seaweed added into cream cracker formulation.

Along 35 days of storage at room temperature, the biscuits samples that presented the lowest firmness at the beginning of storage, a noticeable increase was observed on enriched cream cracker B7, B10 and B15. This increase in biscuits texture might be a response to available compounds from the brown seaweed. However, the biscuits samples with the highest concentration level of seaweed

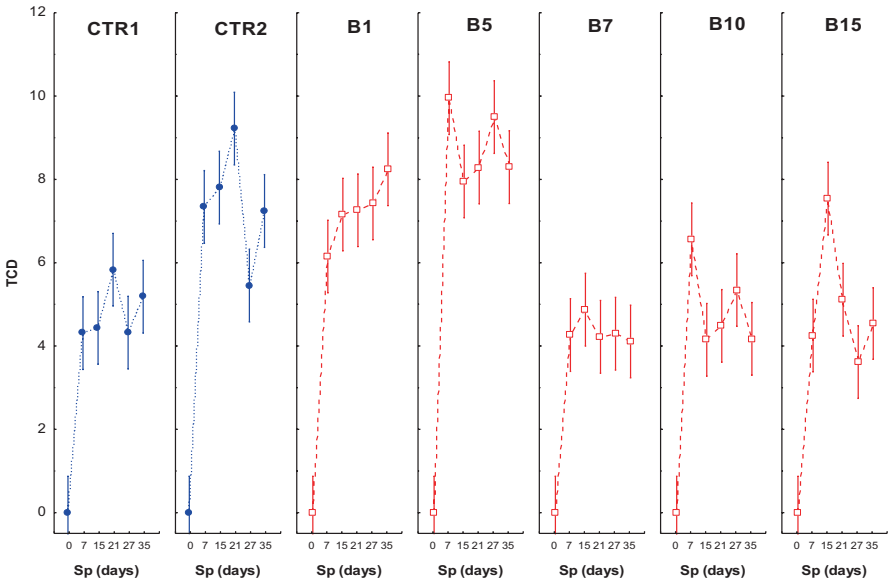

Fig. 9.6 Total color difference of cream cracker samples without (CTR1, CTR2 as a standard cream cracker and without salt, respectively) and with aqueous extracts of Kombu seaweed (B1, B5, B7, B10, B15 with 1%, 5%, 7%, 10% and 15% of aqueous extracts, respectively) during 35 days of storage. Vertical lines denote standard deviation. ● Control cream cracker samples, □ Enriched cream cracker samples

aqueous extracts did not reveal this phenomenon, a pronounced decrease of maximum force was detected.

The lowest concentration level of aqueous extract of Kombu seaweed was the less effective on maintaining the biscuit firmness for 35 days, leading to a value of maximum force inferior of 2 N at the end of storage. On the other hand, the highest concentration level of seaweed aqueous extract demonstrated a higher and remarkable firmness when compared to other samples. Kulkarni and Joshi (2013) refereed that protein and carbohydrates are two important nutrients and contribute to biscuits hardness.

9.3.3 Effects of Aqueous Extracts of Kombu Seaweed in Antioxidant Capacity and Phenolic Content of Cream Cracker

In recent years demand for natural antioxidant sources has been increased, being the seaweed a promise to be used as ingredients not only in food but also the cosmetic and pharmaceutical industry (Marinho et al. 2019).

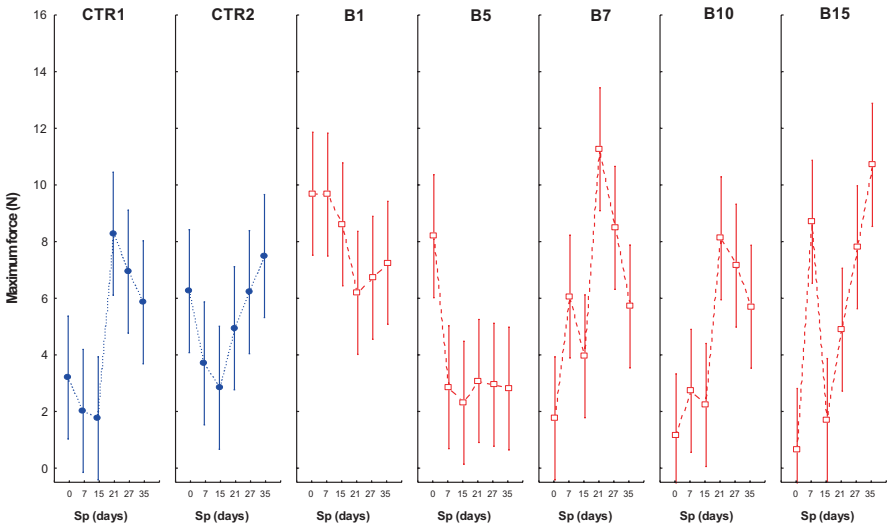

Fig. 9.7 Firmness of cream cracker samples without (CTR1 and CTR2 - cream cracker standard and without salt, respectively) and with aqueous extracts of Kombu seaweed (B1, B5, B7, B10, B15 with 1%, 5%, 7%, 10% and 15% of aqueous extracts, respectively) during 35 days of storage. Vertical lines denote 95% confidence intervals. ● Control cream cracker samples, □ Enriched cream cracker samples

The antioxidant capacity expressed by DPPH scavenging activity of control and enriched cream cracker with aqueous extracts of Kombu seaweed is shown in Fig. 9.8.

The DPPH scavenging activity of CTR1 and CTR2 control samples exhibited a similar value, 31.71% and 29.51%, respectively. On the other hand, the enriched cream cracker samples with 10% and 15% of aqueous extracts of Kombu seaweed revealed a higher and significant ($p < 0.05$) antioxidant capacity compared to both CTR samples, 39.16% and 38.92%, respectively.

Based on previous studies, the antioxidant capacity of seaweed depends on many factors, such as the polarity of extraction solvents, as reported by Koivikko et al. (2005) where the phenolics compounds from brown seaweed *Fucus vesiculosus* augment with solvent polarity. Also, the antioxidant capacity in seaweed is associated with phenolic content (Roleda et al. 2019).

The effect of aqueous extracts of Kombu seaweed in total phenolic content of cream crackers can be observed in Fig. 9.9.

The polyphenols content of enriched cream crackers with seaweed aqueous extracts ranged from 6.91 to 9.00 mg GAE.100 g^{-1} in B1 and B15 biscuit samples, respectively. In both control samples, an identical ($p < 0.05$) phenolic content was achieved (6.70 and 9.29 mg GAE.100 g^{-1}, in CTR1 and CTR2, respectively) compared to enriched biscuits at beginning of storage period.

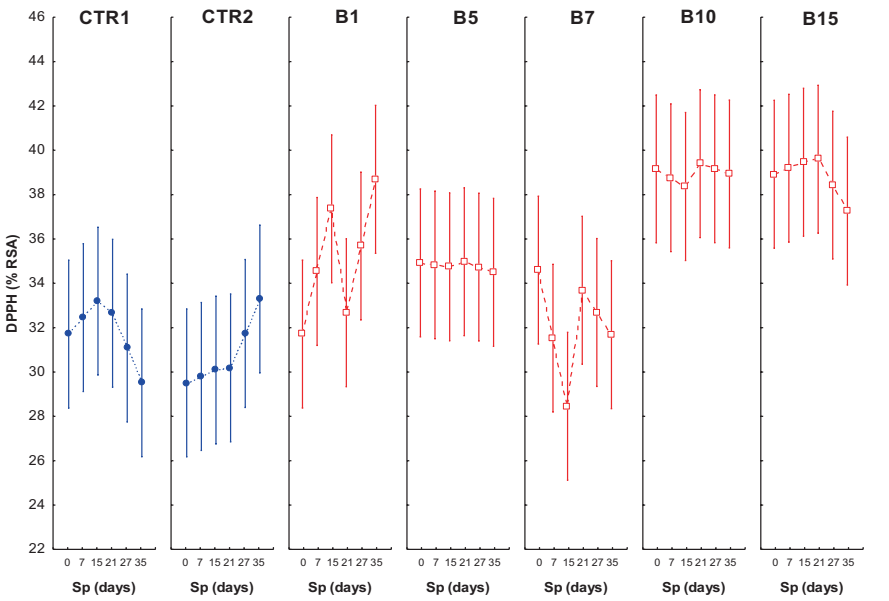

Fig. 9.8 Antioxidant capacity expressed by DPPH radical scavenging activity (%RSA) of cream cracker without (CTR1 and CTR2 - cream cracker standard and without salt, respectively) and with aqueous extracts of Kombu seaweed (B1, B5, B7, B10, B15 with 1%, 5%, 7%, 10% and 15% of aqueous extracts, respectively) during 35 days of storage. Vertical lines denote 95% confidence intervals. ● Control cream cracker samples, □ Enriched cream cracker samples

The highest phenolic content in enriched biscuits samples was attributed to the concentration of seaweed aqueous extract of 7%, 10% and 15% reached the values of 17.99, 15.10 and 16.85 mg GAE.100 g^{-1}, respectively, at the end of storage.

However, the decrease of total phenolic content in all cream crackers could be associated to the heat of the baking process, since some compounds had higher sensitivity to heat, as mentioned by Mahloko et al. (2019). Regarding the study developed by Krystyjan et al. (2015) the decrease of the phenolics content of baked food products is associated with the depolymerization of polyphenols and decarboxylation of phenolic acids occurred in baking treatment. Also, the Maillard reaction could contribute to the decrease of these compounds (Gelinas and McKinnon 2005).

9.4 Conclusions

The addition of aqueous extracts of Kombu seaweed influence the overall quality of cream cracker formulation. Color and texture, two important quality attributes showed an improvement by enrichment of brown seaweed aqueous extract. Also,

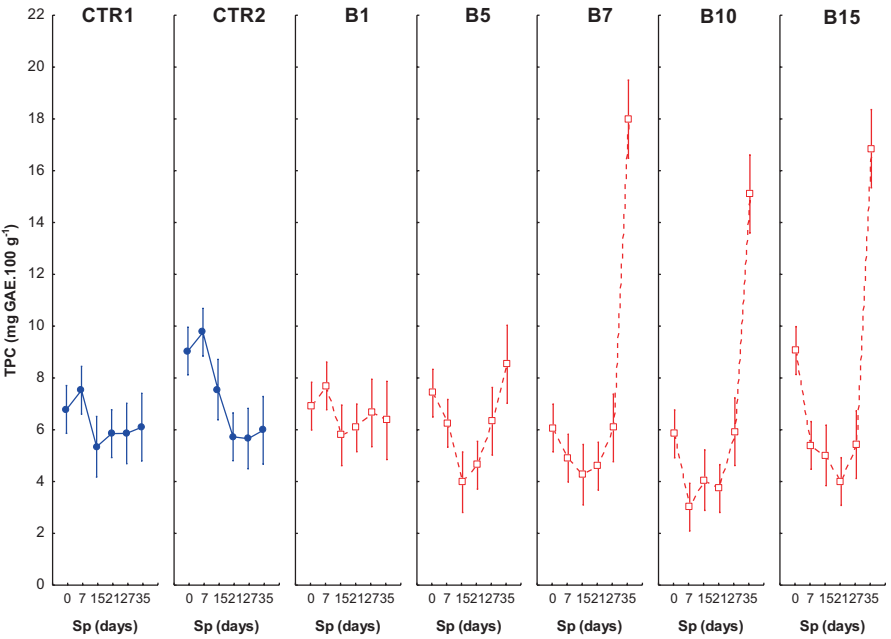

Fig. 9.9 Total phenolic content (TPC, mg GAE.100 g^{-1}) of cream cracker group samples without (CTR1 and CTR2 - cream cracker standard and without salt, respectively) and with aqueous extracts of Kombu seaweed (B1, B5, B7, B10, B15 with 1%, 5%, 7%, 10% and 15% of aqueous extracts, respectively) during 35 days of storage. Vertical lines denote 95% confidence intervals. ● Control cream cracker samples, □ Enriched cream cracker samples

the Kombu seaweed is a natural and good source of antioxidants compounds and in this light, the process of aqueous extracts provides the availability of these compounds which can be used for the enrichment of cream cracker. So, the addition of this seaweed aqueous extract in biscuit formulation has a greater potential in overcoming health consumers by food nutrition. Considering the total phenolics content of all enriched biscuits samples, the high concentration level of aqueous extracts, 7%, 10% and 15% were found to be the best. The findings of our study enhance the interest in using a natural and potential source of antioxidants for the development of new food product—cream cracker that intends to reach to all people in the world.

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Part V
Valorization of By-Products from the Food Industry

Chapter 10
Non-compliant Fruit as New Functional Food Ingredients

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10.1 Introduction

In recent years, healthy eating habits were recognized as fundamental due to the beneficial properties in human health associated with the high level of bioactive compounds (BCs). In this context, consumption of fruits assumes great relevance, since they are rich in these BCs with antioxidant, anti-diabetic and anti-inflammatory activities (Gómez-García et al. 2020). Currently, the main access of consumer to the fruit is restricted to the supermarkets, where the fruit is presented with good aesthetic requirements and under standard size. So far, fruit with a non-standard size or with small irregularities on the surface are usually restricted on the acceptance, assuming a classification of non-compliant fruit that ends frequently as fruit losses in the producer. However, due to their richness in bioactive compounds, non-compliant fruit should not be discarded, but instead considered as value-added value coproduct. Therefore, an effort to find value-added for these fruits has been made contributing at the same time to comply with relevant guidelines of the European Commission and National government that promote food losses and waste reduction or reuse. Efficient waste management is among the most important challenges yet to be addressed in the twenty-first century, and both meet up in the agri-food sector. The production of new functional ingredients from these fruits consists of a valuable and sustainable approach for taking advantage of these food losses. Thus, this book chapter aims to present the most promising alternatives for the valorization of non-compliant fruit, currently underexploited, as well as the challenges that may arise when considering the valorization of this waste in the form of value-added bioactive products.

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10.2 Food Losses and Waste

The Food and Agriculture Organization of the United Nations (FAO) estimates that, annually, about one-third to half of all food globally produced for human consumption is either lost or wasted, leading to considerable economic (representing a cost of around 990 billion dollars), social and environmental costs (FAO 2011; Trigo et al. 2019). Of these, in 2012, more than 88 million tons per year were generated within the European Union (Jiménez-Moreno et al. 2019). Therefore, this problem translates into economic losses for farmers and other stakeholders in the food supply chain and into higher prices for consumers. Additionally, hinder the transition to environmentally sustainable food systems and, since they are associated with considerable use of land, water, energy and agricultural inputs, they also contribute to the emission of millions of tons of greenhouse gases (Adam 2015; van Giesen and de Hooge 2019). Food losses occurring mainly in the first stages of the food supply chain (agricultural production and postharvest) refers to the decrease in edible food mass throughout part of the supply chain that specifically leads to edible food for human consumption (FAO 2011). However, food loss occurring at the end of the food supply chain (retail and final consumption) are usually food waste since it were discarded or because it was not consumed by humans, which relates to retailers and consumers practices and behaviors (FAO 2011; Parfitt et al. 2010; Thyberg and Tonjes 2016). In industrialized regions, food is to a significant extent wasted at the consumption stage meaning that it is discarded even if it is still suitable for human consumption (FAO 2011). Studies commissioned by Gustafsson et al. (2013), estimated that yearly global about 50% of the fruit and vegetable production were food losses and waste. In Europe, losses in agricultural production dominate (20%) (Fig. 10.1), mostly due to the grading caused by demanding quality standards (e.g. aesthetic requirements) set by the Europe, retailers and consumers concerning products shape, size and color (FAO 2011; Jiménez-Moreno et al. 2019). Nevertheless, food losses at the end of the food supply chain are also substantial (19%) in the industrialized regions, since society is very consumerist wasting a lot (Baptista et al. 2012; FAO 2011). The causes of food waste vary throughout the agro-food chain and differ from region to region. As mentioned above, part of the generated food waste comes from the demand of society standards. Thus, some of the agricultural products, although tasty and with good quality, are wasted since do not meet certain parameters.

10.2.1 Non-compliant Fruit

These losses generated during production result in a waste ranging from 10 to 30% for farmers, mainly due to its aesthetic requirements and are called non-compliant fruit. Elimination of these residues is complex and, in many cases, requires heavy investment. Therefore, these food losses are usually conducted for animal feed, or

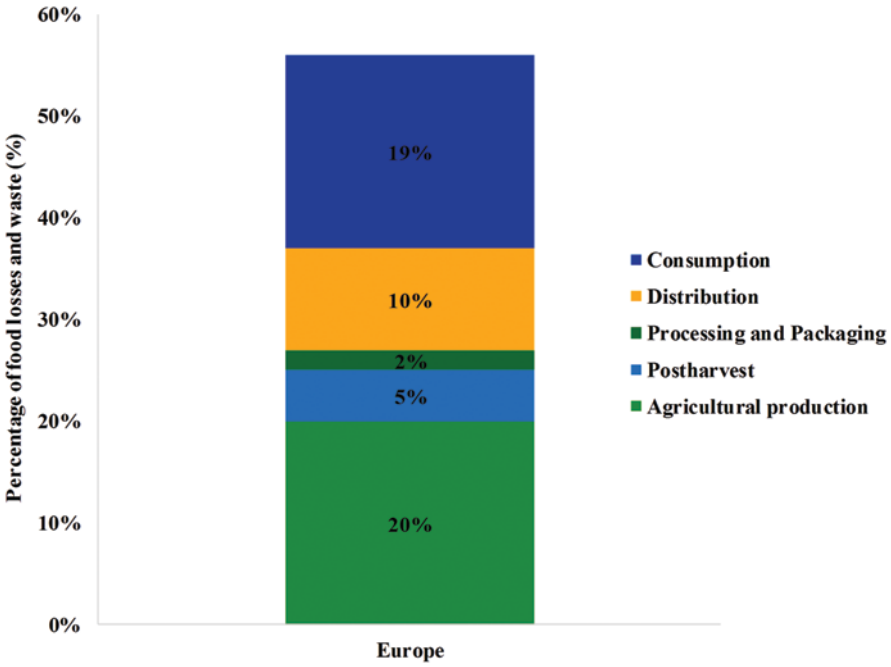

Fig. 10.1 Percentage of food losses and waste of fruits and vegetables generated in each stage of food supply chain in Europe. Adapted from FAO (2011)

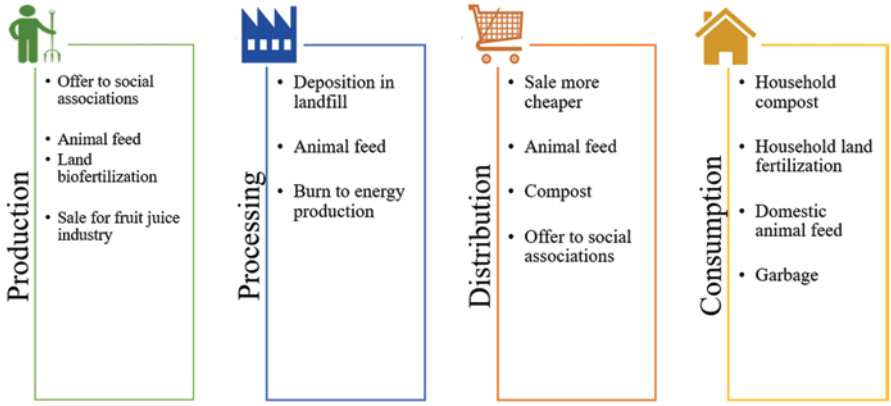

Fig. 10.2 General destination of fruit losses during the food supply chain. Adapted from (Baptista et al. 2012)

to supply social institutions or to supply the production of concentrated juice or to be incorporated into the ground for natural soil fertilization (Fig. 10.2). Losses in processing, distribution and consumption are mainly peels, seeds, pomace and trimmings, and are usually applied, for example, to be burnt towards energy production, sale at cheaper price because the shelf-life is in the end a garbage, respectively.

All the fruit that does not conform to the aesthetic requirements (color, size and shape) and is wasted in production and that is still perfectly edible and safe to eat are non-compliant fruit, previously described. Such fruit represents more than 50 million tons of waste every year in the EU, especially because it does not fit with the aesthetic parameters (Porter et al. 2018). Different factors contribute to the fruit losses as non-compliant food, such as; (1) natural modification on shape or color during production (plant diseases or climate changes), (2) damage during harvest and transportation and (3) low-quality conditions during post-harvest storage. All these considerations directly affect fresh fruit which becomes in economic losses to the producers when they start with the fruit selection process, selecting only those that satisfy the aesthetic requirements (Adam 2015; Göbel et al. 2015; FAO 2011). Supermarkets at the farm gate due to rigorous quality standards concerning weight, color, size, shape and appearance reject some fruit. Therefore, a large amount of fruit never leaves the farms.

Two quality standards for fresh products can be identified in European-regulations: (1) general marketing standards, consisting of minimum quality requirements regarding the freshness, safety and shelf-life of a product and guarantee the consumers' safety and wellbeing and (2) specific marketing standards, applied to only 11 fruits and vegetables and consist mostly of aesthetic requirements (product's color, size and shape). Furthermore, retailers have been maintaining the old and their quality requirements as a guideline and have been reluctant to sell non-compliant fruit, since they assume that consumers are not willing to buy it and because selling this fruit might affect the image of the high quality store (De Hooge et al. 2018; Loebnitz et al. 2015). Then, changing the regulation is not enough to decrease fruit losses. Recent studies from Helmert et al. (2017), De Hooge et al. (2017) and Loebnitz and Grunert (2015), showed that several consumers prefer to buy compliant fruit instead of non-compliant when the priced is equivalent, as expected. This behaviour could be explained by the fact that consumers use the appearance to measure the intrinsic quality of fruit (Göbel et al. 2015; Helmert et al. 2017; Aschemann-Witzel et al. 2015). However, when some retailers have taken the initiative to sell non-compliant fruit at cheaper prices it appears to be effective in increasing sales of this kind of fruit (Aschemann-Witzel et al. 2016; Kulikovskaja and Aschemann-Witzel 2017). Nevertheless, is not a long-term solution to reduce fruit losses. Firstly, because price reduction leads consumers to think that non-compliant fruit have less quality; and secondly, selling non-compliant fruit at low price next to more expensive than normal fruit in the long-term lead to reduction of profits for both retailers and producers (Aschemann-Witzel et al. 2017; De Hooge et al. 2018; Völckner and Hofmann 2007; Adam 2015).

For all these unfavourable reasons different approaches are needed regarding non-compliant food application, which contribute with the world sustainability,

exploiting its nutritional and phytochemical richness developing novel functional food supplies. In this context, a great scientific interest has risen in order to employ the high amount of non-compliant fruit. Consequently, an effort to increase the value-added for this kind of food has been made as well as with the purpose to decrease the food losses generation. However, the production of functional ingredients and/or products is a good alternative to increase the income that comes from these fruits, such as extraction of specific BCs for the production of antioxidant extracts or functional fiber flours. The optimal valorization strategy depends on the nature and properties of the substances that are still present and can be obtained from each fruit (Jiménez-Moreno et al. 2019). Currently, there are a lot of studies on fruit waste, mainly employing peels, pomace and seeds, and such studies showed their abundance of BCs and exposed these raw materials as rich sources of beneficial BCs with health benefits for the consumers and as a cheap and renewable source (Trigo et al. 2019; Jiménez-Moreno et al. 2019). However, there are scarce data available, regarding the employment or valorization of non-compliant food as sources of nutritional molecules and BCs. In Fig. 10.3 are represented the traditional process of fruit industrial production as well as its possible future management. Traditionally, fresh fruit categorized as compliant fruit is distributed by the producers (farmers) to different customers (mostly markets and fruit processing industries) to get the normal (high) economy revenue. On the other hand, the non-compliant food is highly accumulated and categorized as food grade and non-food grade, the food grade is commonly used for juice producers, while the non-food grade is used for animal feeding with lower economic revenue. Currently,

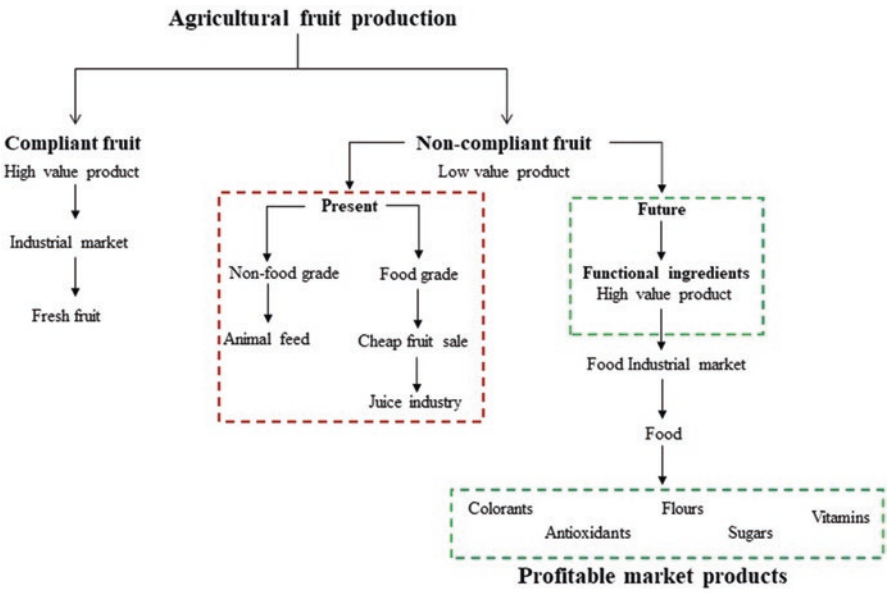

Fig. 10.3 Chart flow of compliant and non-compliant fruit in agricultural fruit production

non-compliant fruit future management could pass through the development of novel supplies, such as functional ingredients, which also can be reincorporated within the industrial chains increasing the industrial profits. Functional ingredients (for example, extracts) obtained from non-compliant fruit are rich in BCs and they can be used in a wide range of products developing of functional foods. Even when the fruit is non-compliant the nutritional quality and microbiological safety are still good and that is why this fruit can be consumed and the health benefits obtained. Fruit production generate a high number of non-compliant fruit, which is perishable due to the high-water content and huge amounts of organic load, as well as their chemical composition, particularly rich in dietary fiber and phytochemicals, provides a costless source of BCs that may favor an efficient and sustainable industrial development. A circular economy model can be implemented for instance in the apple production by recycling its non-compliant production, thereby creating added value with fewer resources. As already mentioned above several strategies for the valorization of these food losses have been proposed, including its direct use for animal feed, but a more sustainable approach should be designed and applied to the apple producers, increasing their economic revenue, as well adding value to the non-compliant fruit products (Fig. 10.3).

10.3 Valorization of Non-compliant Fruit

The non-compliant fresh fruit fractionation is the common approach that generates high-added-value products with applications in several areas. The main objective of the fractionation process is to generate two different fractions: liquid fraction and solid fraction. Thus, compounds with high molecular weight like dietary fiber remain in solid fraction and the smaller and soluble molecules, such as phenolic compounds and simple sugars stay in the liquid fraction. Before starting the fractionation will be interesting to apply a bleaching process as pre-treatment, that could be applied to the fruit, which would allow the reduction of color in the final ingredients due to the inhibition of polyphenol oxidase (Rabetafika et al. 2014). After that, the fractionation process applied could be a simple roll press belt or a press juice centrifuge (cold pressure). In both cases, new functional ingredients/products are produced. These processes are a new low-cost way—process to obtain sustainable and high value-add products within fruit producers' facilities. However, a more complex process could be applied to fractionation by couple extraction processes for specific extraction of phenolic compounds, which are embedded within the cellular matrix of plants, so it is necessary to apply extra extraction methodologies to improve and maximize the recovery yields. However, in this case, it is necessary a pre-treatment, such as wet milling to facilitate and improve the yield of the extraction.

The technologies of extraction can be classified as conventional or non-conventional. Nevertheless, these conventional extraction techniques present various drawbacks, including (1) thermal decomposition of thermolabile molecules

such as phenolic compounds, decreasing the bioactivity of the final extract; (2) high energy consumption; (3) consumption of large amounts of water and/or organic solvents; (4) longer extraction time; (5) low yields (Galanakis 2012). To overcome these limitations, non-conventional extractive methodologies (green methodologies) have been developed to improve these unfavorable facts and they have led to the development of more innovative approaches, with higher yields, low processing times and minimal consumption of extract solvents, water and energy. Green methodologies include ultrasound-assisted extraction, microwave-assisted extraction, pulsed electric fields, supercritical fluid extraction, enzymes assisted extraction and microbial processes (Ferrentino et al. 2018). However, each extraction technique has advantages and drawbacks, so the selection of the most appropriate in each case depends on several factors, mainly availability, acquisition and operational costs, lack of regulatory and well-defined industrial applications.

With the application of this approach, it is possible to obtain different high added value functional ingredients, which will present a different composition and therefore can be applied to different products from different areas. The current state of non-compliant fruit fractionation is outlined in Fig. 10.4. Potential applications of dietary fiber extract due to low-caloric value and functional properties such as water holding capacity, viscosity, gelation and sensory properties may be a good food additive or ingredient, for example, used as wheat flour substitution in bread and other bakery products like cakes and biscuits (Trigo et al. 2019). These replacement increases the fiber content of end-products and brings functional nutritive values. The valorization of the polyphenols existing in non-compliant fruit as new functional food additive increases the antioxidant capacity of the end-products could be used as a natural antioxidant to stabilize lipidic products oxidation once the use of synthetic antioxidants. Furthermore, the polyphenols are linked to numerous health

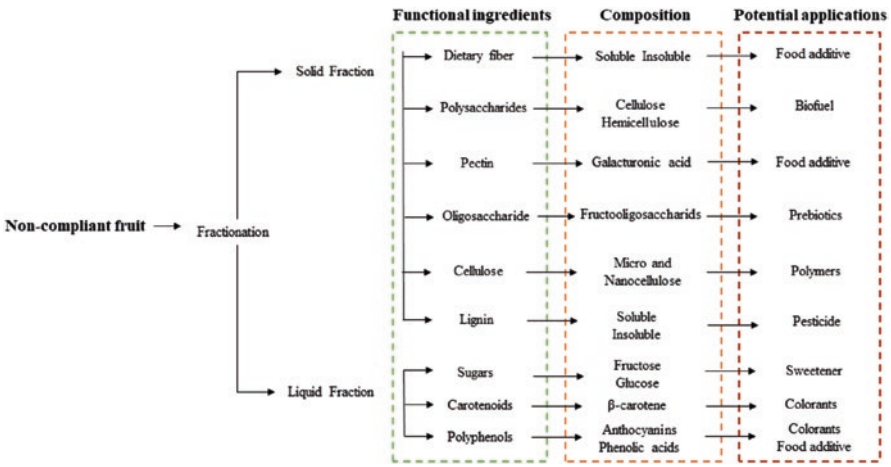

Fig. 10.4 Fractionation of non-compliant fruit into functional ingredients and identification of potential applications

benefits such as antihypertensive, anti-diabetic and anti-cancer activities so, the addition to the food products could provide new functional properties and new perspectives for their commercial use (Campos et al. 2020). Depending of the fruit matrix, the profile, and the composition of the two fractions are different. The dietary fiber, pectin and polyphenols receive the top interest and have been explored for new functional ingredients from food losses, mainly by-products (peels, pomace and seeds) while, other functional fractions (hemicelluloses, cellulose and lignin) are hindered by the complexity of processes (Rabetafika et al. 2014).

10.3.1 Antioxidant Extracts

There are two main groups of BCs: essential and non-essential. The essential BCs include mostly vitamins and minerals and are essential in the prevention of diseases and maintaining specific biochemical processes in the body. While, non-essential BCs consist of secondary metabolites such as phytochemicals, which allow the maintenance of optimal cellular health, leading to an improvement in human longevity (Trigo et al. 2019). These secondary metabolites are extra nutritional constituents that naturally occur in minor quantities in the plant kingdom. Nevertheless, these phytochemicals have received increasing interest in the last years, since they can be found in great amounts in fruit, vegetables, plant-derived beverages, leaves, seeds and peels. Most common phytochemicals include alkaloids, terpenes and phenolic compounds (Martínez-Ávila et al. 2012).

Phenolic compounds are one of the biggest and widely distributed groups of phytochemicals. They are classified in different groups, which include flavonoids, phenolic acids and tannins among others. Therefore, phenolic compounds have common characteristics, namely, the presence of at least one aromatic ring hydroxyl-substituted, commonly bound to other molecules, frequently to sugars (glycosyl residue) and proteins. Flavonoids constitute the largest group of plant phenolics accounting for over half of the thousand naturally occurring phenolic compounds (Martins et al. 2011). Flavonoids are further divided into several categories depending in substitution patterns to ring C in the structure, such as flavones, flavonols, flavanones and flavonols and anthocyanidins. Anthocyanins are glycosylated anthocyanidins, which are water-soluble glycosides and possess several hydroxyl groups, being highly correlated with the antioxidant activity (Gonçalves et al. 2018). On the other hand, the phenolic acids are simple molecules divided into two groups that include hydroxybenzoic and hydroxycinnamic acids. The most frequent hydroxybenzoic acids include gallic, *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acid, while hydroxycinnamic acids, caffeic, ferulic, *p*-coumaric and sinapic acids. Tannins are phenolic compounds of molecular weight from intermediate to high (500–3000 Da) and can be classified into two major groups: hydrolyzable tannins and non-hydrolyzable or condensed tannins. The condensed tannins are polymers of catechin, not readily hydrolyzed by acid treatment, and constitute the main phenolic fraction responsible for the characteristics of astringency of the vegetables.

Although the term condensed tannins are still widely used, the chemically more descriptive term "proanthocyanidins"has gained more acceptance.

The non-compliant fresh fruit fractionation generates a liquid fraction, rich mainly in phenolic compounds well known for their antioxidant activity. Due to their bioactivity, this fraction could be used as a food additive, such as preservative. The preservatives group is divided into three classes, antimicrobials, antioxidants and anti-browning agents (Carocho et al. 2018). The antioxidant is used to extend the shelf life of foodstuffs from the oxidative process that could result in degradation of food, such as lipidic peroxidation, changed the flavor, color, nutritional and texture value, as well as, creation of toxic compounds. For, itself, antioxidant extracts are one of the most important conservation technologies used by the food industry, with their main function being the prevention of oxidative processes (Faustino et al. 2019). Natural antioxidants have been widely studied on the food additives field as an alternative to the synthetic antioxidants since the latest raised issues over the safety application into foodstuffs (Franco et al. 2019). The synthetic antioxidants have been increasingly substituted by natural antioxidants mainly phenolic compounds since in Europe, the use of some of these compounds had been prohibited, and the discovery for new and natural ones has been promoted, subsequently, much of the research on natural antioxidants also focused on phenolic compounds (Campos et al. 2020). Fruit, vegetables, and their by-products are among the most relevant potential sources of natural antioxidants that could be exploited for antioxidant extracts production. Due to the high content of phenolic compounds present in non-compliant fruit, these food losses may be considered as a possible source of new antioxidant extract. Furthermore, is a cheap source of new natural antioxidants and, some of the antioxidant compounds naturally found in some by-products are already approved for use as antioxidant additives and possess an E number, namely ascorbic acid (E300), lutein (E161b), tocopherol (E306), and carotenoids (E160a–E161g) (Carocho et al. 2018).

10.3.2 Dietary Fiber Extracts

Dietary fiber (DF) intake has shown several health benefits, including the regulation of intestinal transit, risk reduction of obesity, diabetes and cardiovascular diseases, as well risk reduction of hyperlipidemia, hypercholesterolemia and hyperglycemia, since modulates food ingestion by influencing digestion, absorption and metabolism of nutrients (Macagnan et al. 2015). DF refers to a group of substances in plant foods that cannot be completely broken down by human digestive enzymes; the common structural base are carbohydrates and their derivatives. The DF are divided into two groups, soluble and insoluble dietary fiber. The soluble DF includes non-starch polysaccharides, such as pectin's, β-glucans, gums, mucilage's, oligosaccharides or inulin and insoluble dietary fiber includes mainly cellulose and hemicellulose (Burton-Freeman 2000; Quirós-Sauceda et al. 2014). Soluble DF has several beneficial physiological functions: (1) increase the viscosity of food, changing the rate of

nutrient release and absorption in the gastrointestinal tract; (2) lowers blood cholesterol concentration since it binds to bile salts in the small intestine leading to excess fecal excretion of bile salts. In contrast, insoluble DF has less physiological effects in the upper gastrointestinal tract. Nonetheless, plays an important role in intestinal regulation by mechanical peristalsis (Trigo et al. 2019). The DF also enclose attached an appreciable number of phenolic compounds, proteins or other substances with positive health effects, which usually co-exists in all plant cell structures (DeVries 2004). Some extraction techniques do not allow the complete extraction of these BCs of a given matrix. In fact, there is a high amount of phenolic compounds that may be disregarded as they remain within the extraction residue (Silva et al. 2018). These compounds are referred to as non-extractable phenolics (NEP). Given their association with these cellular structures, after ingestion, they are not released from the food matrix by any of the steps of the digestive process. Instead, they pass relatively intact throughout the digestive tract, not being absorbed in the small intestine, until they reach the colon where they can be fermented by the gut microbiota (Silva et al. 2018; Trigo et al. 2019). So, NEP compounds and DF are intimately associated; in fact, the phenolic compounds are frequently associated with DF and, should be considered as a specific DF: the antioxidant dietary fiber, which has specific health-promoting properties. Recently, fruit by-products (peels and pomaces) have been discovered as a novel source of DF and are the main source of pectin, gums and mucilage (Campos et al. 2020). Then, non-compliant fruit could be also a novel source of DF, which can be incorporated into market food products. Fruit DF have more quality than cereals DF (majority DF source in food products) due to their higher total and soluble fiber content, water and oil holding capacity and colonic fermentability, as well as their lower phytic acid content and caloric values. For instance, production of DF concentrate powders from non-compliant fruit could be a great commercial opportunity to create new functional ingredients from a renewable and economical source moreover, an environmental problem could be solved.

10.4 Development of New Value-Added Products

As previously described, non-compliant fruit presents a relatively high content of dietary fiber, pectin, polyphenols and antioxidant properties when compared to other sources. These features could be fully exploited for the development of different food ingredients such as supplements, additives or ingredients, which could be potentially applicable in bakery, dairy, beverage and animal products and animal feed industries. The quality and safety of such ingredients could be significantly improved by the addition of bioactive fractions, however, is essential to select the best concentration to keep, without any modification, the sensorial properties regarding texture, color and taste, among others. Diabetes and its drawbacks are a serious global concern, which is progressively developed by the oxidative stress, this health problem is inflicted by the imbalance between the overproduction of

reactive oxygen spices and the short intake of antioxidants (Ibrahim 2017). Polyphenols and carotenoids are natural antioxidants well-characterized by their capability to minimize the negative impact of the oxidative process caused by the free radicals, thus enhancing the problems associated with the oxidative stress (Lo et al. 2017). In this scenario, functional foods are described as food supplies, which exhibit diverse benefits upon human health, helping in the prevention or reduction of certain illnesses, such as cancers, cardiovascular, inflammatory disorders and diabetes (Gómez-García et al. 2020). Beyond, future perspectives for the valorization of non-compliant food could be applied as functional ingredients, since these plant tissues hold a high concentration of significant nutrients namely dietary fiber and proteins, as well as polyphenols and carotenoids which have been shown a good correlation in the prevention of health diseases. For example, apple fractions present high content of dietary fiber which can be directly used to develop functional flours, due to this natural polymer has been contributed to the modulation of the gut microbiota, such as *Bifidobacterium*, a bacteria associated with colon, stomach, prostate and breast cancer prevention (Veiga et al. 2020). On the other hand, polyphenols exhibit good digestive enzymes inhibition, such as lipase, amylase and glucosidase, minimizing hyperglycemia, which is also strongly linked to diabetes and obesity disorders (Sulaiman and Ooi 2014). By these functional benefits and based on their richness in BM, apple fractions could be employed as functional ingredients, developing new food supplies such as flours, fiber bars, or extruded products, which promote health benefits.

10.4.1 Application of New Value-Added Products in Food Supplies

10.4.1.1 Bakery

Bakery supplies are widely consumed worldwide, which are made principally by white wheat or corn flour, as well as sugar, egg, fat, milk and water. All of these ingredients display an important function in the batter and despite being a good source of energy and nutrients, in many cases, they exhibited low antioxidant activity (Quiles et al. 2018). Hence, different studies have been carried out regarding the incorporation of fruit by-products to enhance fiber and antioxidant content. De Toledo et al. (2017) substituted a portion of wheat flour in cookies with flour prepared by pineapple stem, melon peels and apple pomace, where cookies made with 15% of melon flour represented a good relation to the nutritional characteristics about fiber (4.67–6.46%) and ash (1.74–2.25%) contents, while by pineapple and apple flours were positively influenced concerning consumer's preference. Mir et al. (2017) incorporated apple pomace as flour at increasing levels (0, 3, 6 and 9%) for the formulation of gluten-free crackers prepared with rice flour. At 9% of dosage was observed a significant increase in dietary fiber content from 3.01 to 7.41% and soluble dietary fiber from 0.25 to 3.07%. Also, total phenolic content (TPC) and

total flavonoid content (TFC) significantly increased from 0.61 to 0.82 mg GAE/g and 41.96 to 53.88 μg catechin equivalents/g, respectively, with the increase of apple flour levels. Simultaneously the antioxidant capacity increased by exhibiting an increase on DPPH scavenging capacity from 51.7 to 61.53%. Enhancing the fiber content and antioxidant properties of bakery supplies has been an important science trend due to it is well correlated with several health benefits alongside increases in dietary fiber and polyphenols.

10.4.1.2 Beverages

Traditionally, fruit juices are the principal way of significant consumption of bioactive compounds and hence can be used as vehicles to deliver functional health benefits. Beyond, consumers' demand has been increasing for food, not for safe food but also for providing health benefits. In this scenario, some researchers have been focused on novel strategies to development natural additives to incorporate in juices such is the case of Adiamo et al. (2018), showed that the addition of orange by-products extracts to carrot juice enhanced its phenolic content (30.25 mg GAE/100 mL) and antioxidant properties (61% DPPH scavenging activity).

All of these pieces of evidence reinforce the capability to use non-compliant fruit as novel functional ingredients, which positively influence nutritional and biological properties aimed to enhance human health. In general, the new functional ingredients obtained from the valorization of non-compliant fruit can be included at significant levels in many food supplies, while maintaining consumer acceptability, the healthiness of the products and potentially reincorporation in the food chains.

10.4.1.3 Dairy

One of the most significant drawbacks of dairy foods is related to the high content of fat, which is highly susceptible to lipid oxidation. Therefore, the addition of bioactive antioxidants from the fruit into the dairy matrices has been shown interesting good results to retard oxidative processes, improve self-life and increase nutritional aspects (Özvural and Vural 2011). Marchiani et al. (2016) reported an increase in the TPC (55%), antioxidant activity (80%) and acidity (25%), compared with the control, in 3 week-stored yogurts enriched with grape flour (60 g/Kg). For instance, when apple fractions (0.5 and 3%) were directly added in ice cream, fat (10.18%) and protein (4.03%) content was higher than the control (9.48 and 3.67%, respectively) and with high acceptable sensory properties (Ayar et al. 2018). Adhikari and Bajracharya (2018) incorporated apple pulp (6%) in probiotic yogurt, and results showed good self-life after 21 days of storage at 4 °C. Dairy products fortified with apple fractions as natural preservatives can improve the nutritional profits of the final products.

10.4.1.4 Animal Feed and Supplies

Animal feeds are frequently composed of agricultural by-products, such as rice bran, corn, soybean meal or fish meal as complex nutrients. Regarding consumers' demand for attractive food products, aquaculture and chicken feed usually contains bioactive compounds such as fiber, proteins polyphenols and carotenoids. In the animal feed industry, carotenoids are employed as a feed additive for fish and laying hens to enhance the color of their flesh and egg yolk. However, this pigment used in these industries is mostly chemical synthesized which means expensive processes and low yields (Roadjanakamolson and Suntornsuk 2010). Some researchers have been looking for different sources rich in bioactive compounds to employ as animal feed additives, such is the case of Abdollahzadeh et al. (2010), they incorporated a mixture of tomato and apple into alfalfa at different levels (0, 15 and 30%) for feeding dairy cows. A dosage of 30% of tomato-apple pomace with a ratio of 50:50 showed higher content on cow's milk as well as improvement on milk yield production from 20.8 to 21.6 kg/day, compared with the control. On the other hand, animal supplies such as meat, fish and poultry have some inconvenient issues related with their high susceptibility to oxidation and microbial contamination since they present significant content of water, lipids and proteins which face with short self-life and short time of storage, as well as a negative modification on the sensory characteristics. Moreover, natural antioxidants derived from fruit and vegetable have been proven to be efficient and profitable compounds to avoid these drawbacks. Biswas et al. (2015) added to chicken and turkey meat different concentrations of drumstick leaf powder, aloe vera gel, apple and banana peel paste in a concentration of 0.5, 2, 2 and 2.5 g/100 g, respectively. After 60 days of storage at 37 °C, all the treatments had higher antioxidant activity on DPPH, ABTS and superoxide anion scavenging, than the control, where apple peel contained the highest amount of TPC and TFC (18.7 and 8.74 g GAE/100 g, respectively). The application of apple pomace for animal feed as additives or ingredients and as natural meat preservatives could represent a sustainable way to valorize food by-products.

10.5 Conclusion

The current chapter shows a clear example of non-compliant fruit products application with high value-add, which shows the potentialities lost until now as a rich source of bioactive compounds, such as polyphenols and dietary fibers. It is possible to extract these bioactive compounds from non-compliant fruit in a successful way and to exploit their great potential in relevant industries through the production of functional ingredients/foods. The use of non-compliant fruit would allow saving large amounts of fruit, while simultaneously reducing the negative impact on the environment, as well as, increasing the diversity of new ingredients in the market and consequently the availability of new food products, leading to increase of the

economic value of the raw materials and stimulate the transition of fruit producers to the circular economy.

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Chapter 11
Potential of Red Winemaking Byproducts as Health-Promoting Food Ingredients

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11.1 Introduction

The winemaking industry produces huge amounts of wastes (Nunes et al. 2017) being a sustainable source of natural health-promoting compounds (Drosou et al. 2015). The winemaking waste, called grape pomace, is composed by seeds, skins and stems. It represents an environmental problem when incinerated or discarded in landfills, causing pH decrease by phenolic compounds and degradation resistance, as well as attraction of flies and pests, foul odor, pollution and oxygen depletion of water because of tannins and other compounds (Drevelegka and Goula 2020). Grape pomace is a sustainable source of antioxidants and dietary fiber (Rivera et al. 2019). Grape phytochemicals are represented by a wide variety of bioactive compounds such as flavonoids (anthocyanins and proanthocyanidins), simple phenolics mostly derivatives of hydroxybenzoic acid (p-hydroxybenzoic, gallic, gentisic, and

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protocatechuic acids) and hydroxycinnamic acid (ferulic, p-coumaric, caffeic, and sinapic acids), stilbenes, and vitamin E (Georgiev et al. 2014). The main polyphenols found in grapes are flavonoids that present numerous properties such as antioxidant, anti-inflammatory, cardioprotective, neuroprotective, antimicrobial and antiaging (Georgiev et al. 2014; Magrone and Jirillo 2010).

Red winemaking process consists of de-stemming, pressing of grapes to release the juice (must), maceration to extract anthocyanins and tannins from skin and seeds, fermentation in tanks, settling, clarification, filtration and maturation. In the case of red wines, fermentation is done in the presence of the entire grape (juice and pomace) causing anthocyanins extraction, conducted at 28–30 °C, from the skin which gives red wine its typical color, consisting of yeasts conversion of sugar into alcohol for one or two weeks. The process is followed by pressing of the skins to extract the remaining juice and wine obtaining the winemaking residue (20–26% of grape pomace are seeds), followed by a secondary bacterial fermentation (optional) that can decrease the acidity and soften the wine taste through the conversion of malic into lactic acid. Then, settling, clarification and filtration of the wine is followed, as well as maturation which varies depending on the wine (Barba et al. 2016; Beres et al. 2017; Ribereau-Gayon et al. 2006). The polyphenolic content and profile of wine depending on the grape variety, geographic localization of vineyards, viticulture practices, and the winemaking process itself (Fourment et al. 2017; Markoski et al. 2016; Pérez-Navarro et al. 2019). White wine is obtained by the fermentation of grape juice by contrast with red wine production where the fermentation occurs on grape juice in contact with berries (skin, seeds, and stem) (Markoski et al. 2016). In consequence, the polyphenolic content of red wines is higher than in white wines, also possessing different polyphenolic profiles (Magrone and Jirillo 2010; Nardini and Garaguso 2018). Red grape color, which gives the color to red wine, is confided by anthocyanins in the skin: cyanidin, petunidin, delphinidin, peonidin, glucosides [malvidin 3-glucosides, 3-(6-acetyl)-glucosides and 3-(6-p-coumaroyl)-glucosides, peonidin and malvidin 3-(6-caffeoyl)-glucoside]. During the winemaking process, anthocyanins suffer a list of reactions (oxidation, hydrolysis, cycle-addition, condensation and polymerization) where the yeasts reabsorb and fix them onto the solid parts of the grapes such as skins. Other polyphenols, such as tannins, are the compounds responsible for astringency and bitterness (Pérez-Navarro et al. 2019). Most of the polyphenols (70%) remain in the whole by-product (grape pomace) after fermentation (Beres et al. 2019; Da Porto et al. 2015), offering a great opportunity to be used as a healthy food ingredient, natural colorant and/or preservatives to extend food products shelf-life (Beres et al. 2019; Iriondo-Dehond et al. 2018). Red wine polyphenols have shown to maintain immune system homeostasis in the host by releasing pro-inflammatory and anti-inflammatory cytokines as well as nitric oxide, inhibiting atherogenesis and preventing age-related diseases as a consequence of its immunomodulation properties (Magrone and Jirillo 2010).

Non-communicable chronic diseases are the main cause of death worldwide (71% of all deaths), which include cardiovascular diseases, cancers, respiratory diseases, and diabetes, according to the World Health Organization (WHO 2019).

These diseases are the main cause of "premature" deaths between the ages of 30 and 69 years. Metabolic risk factors such as high blood glucose, pressure, and lipids as well as obesity may lead to cardiovascular disease, which is the main chronic disease. These chronic diseases could be prevented by a healthy diet and lifestyle accomplished by routine physical activity (WHO 2019). Among the main metabolic disorders, there is glucose intolerance, insulin resistance, dyslipidemia, and overweight, which may be promoted by chronic oxidative stress and inflammation. The presence of lipids excess leads to the accumulation of fat in the adipose tissue (fat storage endocrine organ composed of adipocytes) delivering chronic low-grade inflammation by other cell types such as macrophages that are promoters of inflammation and oxidative stress. Hormones and cytokines (adipokines) are produced by the adipose tissue and could lead to an overproduction of ROS (Rebollo-Hernanz et al. 2019). Thus, obesity can lead to cellular oxidative stress and insulin resistance, cytokines release, lipid-induced impairment, and dysfunctional protein tyrosine phosphatase signaling leading to the pathogenesis of type 2 diabetes. During oxidative stress, ROS production can cause DNA damage, cell dysfunction, and organelle injury. Moreover, insulin resistance and cell dysfunction can lead to partial or total insulin deficiency, with subsequent development of type 2 diabetes. Glucose and lipids uptake excess, oxidative stress, inflammation, adipokines, and altered insulin secretion could lead to insulin sensitivity (Xu et al. 2018). As already stated, dietary polyphenols possess numerous bioactive properties that may help in the prevention and/or treatment of these diseases and their complications (Xu et al. 2018). In this sense, red grape by-products (pomace, skin, seeds, and stems) are a rich source of polyphenols, especially anthocyanins that may exert several bioactive properties and cope with prevention/treatment of chronic diseases. Grape pomace in general represents approximately 20% of grapes' fresh weight leading to the enormous accumulation of this winemaking industry by-product despite its common use as animal feed or for grape seed oil, citric acid, and anthocyanins obtaining (Martín-Carrón et al. 2000). Thus, the valorization of winemaking by-products for human nutrition and health is of great importance.

11.2 Winemaking By-product Composition

11.2.1 Polyphenols

The intake of polyphenols is associated with the reduction of risk of chronic diseases such as heart disease, atherosclerosis, cancer and diabetes (Nash et al. 2018; Toaldo et al. 2015). These bioactive compounds may exert its antioxidant activity by scavenging oxidant molecules inside (mitochondrial ROS) and outside the cell, interacting with cell membrane proteins and lipids as cell enzymes (modifying their activity because of receptor-ligand binding) and transcription factors (DNA binding site) by the uptake of phenolic compounds into the cells (Hatia et al. 2014).

During red-winemaking polyphenols are extracted from the berry into grape juice. A study on Syrah, Marselan and Tannat wines showed that p-coumaroylated anthocyanin proportions were 5% in wines, 37% in pomace, and 19% in skins, caffeoylated anthocyanins presented higher concentrations in pomace than in skins (synthesis could take place during vinification), di-methoxylated based anthocyanins increased their relative contribution in pomace and wines compared to skins, and for the first time, an anthocyanin acylated with ferulic acid was found in wine in the Tannat samples (malvidin 3-feruloyl-glucoside) (Favre et al. 2019).

Grape pomace has a total polyphenol content of 4.8–5.4% dry matter, but only 2% of them are extractable under mild conditions (Yu and Ahmedna 2013), most of which are highly polymerized condensed tannin, and others may interact with fiber being non-extractable unless strong acidic extraction is performed (Fernández-Fernández et al. 2019; Yu and Ahmedna 2013). Grape pomace is composed of anthocyanins (delphinidin, malvidin, cyanidin), flavanols (epicatechin, catechin, epigallocatechin, gallocatechin), flavonols (quercetin, kaempferol and myricetin), phenolic acids, stilbenes (resveratrol) (Beres et al. 2017), and dietary fiber (Ajila and Prasada Rao 2013; Drevelegka and Goula 2020), including extractable phenolic antioxidants such as phenolic acid, flavonoids, procyanidins and resveratrol from grape seeds, and abundant anthocyanins from grape skins (Drevelegka and Goula 2020; Yu and Ahmedna 2013). Among grape pomace anthocyanins it can be found 3-O-monoglucosides and acetyl glucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin, being malvidin 3-O-glucoside the main anthocyanin (Yu and Ahmedna 2013). Extracts (ethanol: water, 40:60 v/v) of 4 grape pomaces from *Vitis vinifera* (Cabernet Sauvignon and Merlot) demonstrated the potential of grape pomace as a source of phenolic compounds with 13 different anthocyanins, presenting antioxidant activity, as well as a rich source of PUFA (Ribeiro et al. 2015). Red grape pomace extracts obtained by conventional, ultrasound-assisted and microwave-assisted extraction revealed higher phenolic recovery for the non-conventional extraction methods (conventional phenolic recovery 5.7–48.6 mg GAE/g of grape skin), presenting ultrasound-assisted extraction the best phenolic recovery. Grape pomace extracts were composed mainly of the anthocyanin malvidin-3-glucoside, followed by quercetin (Caldas et al. 2018). Syrah red grape pomace extract showed higher contents of the anthocyanins peonidin 3-O-glucoside and malvidin 3-glucoside compared to Petit Verdot pomace extract, being malvidin 3-glucoside the main anthocyanin, and also containing phenolic acids (gallic and syringic), procyanidins B1 and B2, catechin, epicatechin, and quercetin 3-β-D-glucoside (Siqueira Melo et al. 2015). A Syrah grape pomace anthocyanin-rich extract (acidified methanol, pH = 4.0) presented 21 anthocyanins being malvidin-3-O-glucoside, malvidin-3-(6″-acetylglucoside) and malvidin-3-(6-O-p-coumaroylglucoside) the main ones (Trikas et al. 2016). An extract of red grape pomace from Portugal (80% v/v ethanol) presented (−)-epicatechin, caffeic acid, and the major compounds syringic acid and (+)-catechin (Tournour et al. 2015). Also, enzymatic assisted extraction of red grape pomace has been reported. Pectinase, cellulase and tannase extraction of Syrah grape pomace was found to enhanced the extraction yield of phenolics, by the release of gallic acid by tannase and p-coumaric acid and malvidin-3-O-glucoside

by cellulase (Meini et al. 2019). Another study of red grape pomace enzymatic assisted extraction with cellulase and pectinase enzymes showed an increase in phenolics extraction by cellulase as compared to pectinase (Drevelegka and Goula 2020).

Grape seeds contain oil (13–19%) with essential fatty acids, protein (~11%), non-digestible carbohydrates (60–70%), tocopherols and beta-carotene (Yu and Ahmedna 2013) as well as nutritional macroelements (K, Na, Ca, Mg and P) and nutritional essential microelements (Fe, Cu, Zn and Mn) (Lachman et al. 2013). Red varieties contain higher amounts of Fe, Cu, Zn, and comparable values of Mn (Lachman et al. 2013). Grape seeds oil contains high amounts of unsaturated fatty acids (Yilmaz et al. 2011). Besides, polyunsaturated fatty acids (PUFA) have also been detected (Ribeiro et al. 2015), mostly present in the seeds (Manna et al. 2015). Grape seeds are a source of proanthocyanidins which possess antioxidant properties and may also have cardioprotective effects (Drosou et al. 2015; Lachman et al. 2013; Yilmaz et al. 2011), cataract prevention, anti-hyperglycemic effects, anti-inflammatory effects as well as anti-cancer efficacy (Drosou et al. 2015). Grape seeds possess monomeric phenolic compounds [(+)-catechins, (−)-epicatechin and (−)-epicatechin-3-O-gallate], and procyanidins (dimeric, trimeric and tetrameric) (Yu and Ahmedna 2013). Grape seeds from red varieties grown in Serbia were found to possess flavan-3-ols as the main phenols, most of which were gallocatechin gallate and catechin (Pantelic et al. 2016). As to red grape variety "Prokupac" seed extract, among phenolic acids ellagic acid was the most abundant, followed by gallic acid, representing a high content compared to other grape varieties seed extract, and among flavonols quercetin and isorhamnetin were the most abundant (Pešić et al. 2019).

Grape skin phenols can be located in the cell wall, bound to polysaccharides through hydrogen bonds and hydrophobic interactions, and can be confined in cell plant vacuoles or associated with the cell nucleus. During red winemaking, the fermentation of grape juice in contact with grape skin and seeds grape skin phenols suffer a mild ethanolic extraction that still leaves grape pomace with a high amount of polyphenols because of skin matrix retention (Pinelo et al. 2006). Grape skin is a source of anthocyanins, flavonols, flavonol glycosides, and hydroxycinnamic acids, whereas gallic acid and flavonols were mainly present in the seed portion (Yu and Ahmedna 2013). Red grape skin shows the highest concentration of tannins (mainly catechin, epicatechin and epicatechin gallate) (Deng et al. 2011) with a greater degree of polymerization and lower quantity of gallates when comparing with seeds, as well as containing other polyphenols such as gallic acid and its glucosides, caftaric and coutaric acid, resveratrol, quercetin and kaempferol glucosides and glucuronides (Pinelo et al. 2006). Red grape skin has higher amounts of reducing sugars, total phenolics, anthocyanin, and resveratrol than pulp (Ni et al. 2017), and its color is due to anthocyanins presence, which can be extracted by an acidic medium, are stable at low pHs and possess antioxidant activity (Fernández-Fernández et al. 2019; Vatai et al. 2009). Resveratrol, also present in red grape skin, passes down to wine during maceration, but a significant amount remains in the pomace (Yu and Ahmedna 2013). Deng et al. (2011) reported a total phenolic content of red wine grape peel was of 21.4–26.7 mg GAE/g dry matter and total flavanol content resulted

in 31.0–61.2 mg CE/g dry matter and proanthocyanidin contents was of 8.0–24.1 mg/g dry matter for five wine grape pomace varieties (two white and three red). Pantelic et al. (2016) found mostly flavonols (quercetin and myricetin) and regarding anthocyanins 20 derivatives of delphinidin, malvidin, cyanidin, petunidin, and peonidin were found in 7 red grapevine varieties grown in Serbia ('Cabernet Sauvignon', 'Merlot', 'Cabernet Franc', 'Shiraz', 'Sangiovese', 'Pinot Noir' and 'Prokupac') (Pantelic et al. 2016). In the case of the red grape variety "Prokupac", skin extract presented a 24.4% of phenolic acids (mainly ellagic acid), 65.9% of flavonols (mainly quercetin and isorhamnetin, and detecting glycosides of quercetin and isorhamnetin), and approximately 6% of anthocyanins of total polyphenol content. Particularly, this grape variety contains moderate amounts of anthocyanins, which after a drying process, yields malvidin-3-O-glucoside as the most abundant anthocyanin, followed by peonidin-3-O-glucoside (Pešić et al. 2019). Among red varieties, Tannat is the richest *Vitis vinifera* cultivar in tannins containing high levels of tannins in seeds, and high levels of anthocyanins in skins at maturity (Da Silva et al. 2013), and Tannat grape skin was reported for the absence of galloylated forms and prodelphinidins ranged between 30 and 35% with very low values for epigallocatechin, as well as identifying eleven phenolic acids in Tannat grape skins and wines (Boido et al. 2011). Studies at different maceration times and different winemaking technics have shown a decrease in anthocyanins post-fermentation concentration mostly by the fixation on yeasts and solids (Boido et al. 2011).

11.2.2 Dietary Fiber

The World Health Organization recommends a 25–30 g daily intake of dietary fiber (WHO 2019). Dietary fiber consists of the "carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine" (AACC Report, 2001). Dietary fiber consumption is associated with reduced risks of cardiovascular disease, cancer, and diabetes (Deng et al. 2011). The ideal ratio of soluble-insoluble fiber for consumption is 1:3. Soluble fibers are known to increase viscosity, reduce the glycemic response and plasma cholesterol, protect against inflammatory bowel diseases and prebiotic effect improving host health, whereas insoluble fibers are known for having low density, high porosity, increasing fecal bulk, and reduced diabetes risk. Soluble fiber can be fermented by large intestine microbiota producing short-chain fatty acids, positively affecting major regulatory systems (blood glucose and lipid levels), colonic environment and intestinal immune functions (Yu and Ahmedna 2013).

Grape pomace has been recognized for many years now as a potential source of dietary fiber. Red grape pomace of a mix of grape varieties was found to possess an acid detergent fiber content of 42.4%, and the neutral detergent fiber content of 48.5%, with a lignin content of 31.9%, cellulose content of 10.5%, and hemicellulose of 6.1% (Gowman et al. 2019). Grape skins' and seeds' main components are insoluble fiber and polyphenolic compounds, containing little

soluble fiber (20–40 g/kg) (Martín-Carrón et al. 2000). Polysaccharides can be associated to polyphenols by covalent, ionic or hydrogen bonds, being recognized as potential antioxidant dietary fiber (Beres et al. 2019). Thus, the extraction of bioactive compounds from the grape pomace constituents' matrix represents a loss of dietary fiber component that promotes health benefits. Plant cell-wall architecture is formed by a rigid network composed of cellulose (a linear polymer of β-1,4-linked glucose) and hemicelluloses (xyloglucans and xylans) that interacts with a gel-like matrix of hydrated pectins (Drevelegka and Goula 2020). Dietary fiber architecture can be visualized as a net that entraps polyphenols and so it may be non-hydrolyzable by digestive enzymes in the small intestine. Consequently, they pass to the large intestine where they can be fermented as well as dietary fiber and so leading to systemic effects because of the production of metabolites such as phenylacetic, phenyl propionic, and phenyl butyric acids, among others. Moreover, they can just create an antioxidant environment in the colonic lumen contributing to the scavenging of free radicals and the amelioration of dietary pro-oxidants effects (Ajila and Prasada Rao 2013; Dufour et al. 2018). A clear example of a polyphenolic linkage to dietary fiber is ferulic acid, which takes part in the binding between polysaccharides and the lignin constituents. Lignin is not a polysaccharide because in its composition appears some acids through enzymatic reactions: ferulic, p-coumaric, diferulic, sinapic, cinnamic and p-hydroxybenzoic. Thus, enzymatic hydrolysis could be a solution to accomplish an effective extraction of cell wall polyphenols (Pinelo et al. 2006), as in the case of pectinase, cellulase and tannase enzymatic assisted extraction from red grape pomace enhancing the release of polyphenols from dietary fiber net (Drevelegka and Goula 2020; Meini et al. 2019). Particularly, p-coumaric acid can be released from the insoluble fraction (attached by ester bonds to lignocelluloses) or the soluble fraction (bound to small molecules by ester linkages and is stored in vacuoles or is in its free form) by cellulase (Drevelegka and Goula 2020). Furthermore, special solvent mixtures (such as acidic solvents and organic solvents: ethanol, methanol) may also achieve polyphenols release as well as green and economically viable alternatives to the conventional techniques such as ultrasound-assisted extraction (Drevelegka and Goula 2020; Fernández-Fernández et al. 2019), through the cavitation phenomenon (formation of bubbles in the liquid that favors solvent penetration) (Drevelegka and Goula 2020) and microwave-assisted extraction (Caldas et al. 2018; Drevelegka and Goula 2020) penetrating the plant matrix and favoring cell rupture by generated heat within the cell (Drevelegka and Goula 2020).

Red grape pomace fiber is mainly composed of cellulose, small proportions of pectins and hemicelluloses, finding different contents depending on the grape variety. 'Tempranillo' red grape pomace possesses 36.9% (fresh weight) and 'Manto Negro' 77.2% (dry matter) (O'Shea et al. 2012). Brazilian Pinot noir grape pomace aqueous extracts obtained in hot water showed the presence of pectic- and glucose-based polysaccharides, composed of Rha:Ara:Xyl:Man:Gal:Glc:GalA in a 3:32:2:13:11:20:19 M ratio (Beres et al. 2016).

Grape skin cell walls present neutral polysaccharides (30%, including cellulose, xyloglucan, arabinan, galactan, xylan and mannan), acidic pectin substances (20% of which 62% are methyl esterified), insoluble proanthocyanidins (approximately 15%), and structural proteins (<5%) (Pinelo et al. 2006). Deng et al. (2011) reported for grape skin that 95.5% of total dietary fiber was insoluble dietary fiber. They determined grape skin insoluble dietary fiber was composed of Klason lignin (7.9–36.1% dry matter), neutral sugars (4.9–14.6% dry matter), and uronic acid (3.6–8.5% dry matter). Total dietary fiber from red grape skin resulted in 51.1–56.3%, and soluble sugars in 1.3–1.7% dry matter, respectively (Deng et al. 2011). Grape skins also contain a small amount of pectic polysaccharides (rhamnogalacturonan I and rhamnogalacturonan II), among which pectin is widely used as a gelling and stabilizing agent and as a functional food ingredient (Beres et al. 2017). Mendes et al. (2013) also reported the chemical composition of red grape skins but in this case for the Touriga Nacional variety (20.8% cellulose, 12.5% hemicelluloses, 18.8% proteins, 13.8% tannins, 5.0% extractives soluble in dichloromethane and 7.8% ash), indicating 26.4% water-soluble compounds, which are mainly monomeric sugars (glucose and fructose), and a complex mixture of hemicelluloses (pectin, the most abundant, and acetylated glucomannan). They also stated that most structural polysaccharides amount, such as cellulose, xylan, xyloglucan and others, is entrapped in the cuticular layer and so being poorly accessible to the acid hydrolysis (Mendes et al. 2013).

11.3 Health-Promoting Properties of the Red Grape By-product

11.3.1 Antioxidant

Antioxidants are molecules that neutralize free radicals preventing the damage of biomolecules that may lead to the development of many chronic diseases. Furthermore, the normal aging process is associated with cumulative oxidative stress and low-grade inflammation (Petersen and Smith 2016). Chronic diseases such as cardiovascular disease (CVD), diabetes, metabolic syndrome, and Alzheimer's disease are associated with advanced age. Aging oxidative stress is mainly driven by reactive oxygen and nitrogen species (RONS), which include superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), peroxynitrite (ONO_2^-), and nitric oxide (NO). These reactive species are produced during normal body functioning (growth, repair, and immune functions) but they can also damage biomolecules. The endogenous enzymatic antioxidant system which includes superoxide dismutase (SOD), glutathione peroxidase, catalases, glutathione/TrxR, and peroxiredoxins, is often surpassed by RONS overproduction or consumption, being necessary dietary antioxidants intake (Petersen and Smith 2016). NADPH oxidase system produces ROS exerting antimicrobial mechanisms but also resulting in

collateral damage to tissues such as the parenchymal cells in the brain (Glass et al. 2010). Particularly, flavonoids are good scavengers of reactive oxygen species (ROS) and up-regulate antioxidant defenses (Xu et al. 2017). Grape by-products have been recognized for having high benefit and low risk to counteract both oxidative stress and inflammation (Petersen and Smith 2016) (Fig. 11.1).

Red grape pomace antioxidant dietary fiber presented an antioxidant capacity determined by DPPH method of 153 ± 9 g of dry sample/g DPPH CE50% and FRAP 525 ± 28 μmol TE/g of dry sample) (Sánchez-Alonso et al. 2006). Red grape pomace intake (5%) has been reported for increasing the antioxidant status of piglets with increased total antioxidant status in the liver, spleen, and kidneys, through the augment of catalase activity (spleen and kidneys), superoxide dismutase activity (liver, kidneys, and spleen), and glutathione peroxidase activity (kidneys), as well as through the decrease in lipid peroxidation (liver and kidneys) (Chedea et al. 2019). Red wine grape pomace has also been reported for increasing plasma antioxidant activity in a murine model of lethal ischemic heart disease (atherogenic diet-fed

Fig. 11.1 Bioactive properties of red grape pomace

SR-B1 KO/ApoER61h/h mice), as well as reducing premature death, and changing TNF-α and IL-10 levels (Rivera et al. 2019).

As previously stated, grape pomace is a source of dietary fiber and polyphenols that interact with each other, making the extraction of grape polyphenols from the berry matrix necessary for the phenolic compounds to exert their bioactivity. Grape pomace enzymatic-assisted extraction has been studied using tannase, pectinase and cellulase enzymes to increase polyphenols release, finding an increase of antioxidant capacity with the release of caffeic acid, gallic acid, quercetin, and trans-resveratrol, mostly in the case of using tannase with 1.8 and 3.7 fold for DPPH and ORAC values as well as a mild increase of FRAP value (Martins et al. 2016). Syrah grape pomace enzymatic-assisted extraction with tannase and cellulase showed a 66% increased total polyphenol content and an 80% increased antioxidant capacity, finding tannase extraction leads to a gallic and syringic acids enriched phenolic extract while cellulase leads to a p-coumaric acid and malvidin-3-O-glucoside enriched one (Meini et al. 2019). In another study, grape pomace enzymatic-assisted extraction followed by ultrasound-assisted extraction with cellulase resulted in 48.76 ± 1.06 mg GAE/g dry pomace (Drevelegka and Goula 2020). Syrah red grape pomace extract (ethanol: water) showed 310 ± 7 μmol TE/g sample for DPPH, 653 ± 34 μmol TE/g sample for ABTS, 1363 ± 79 μmol TE/g sample for peroxyl radical (ROO·), 0.24 ± 0.01 μmol TE/g sample for superoxide radical (O_2^-) and 0.031 ± 0.001 mg/mL (EC50) for hypochlorous acid (HOCl) (Siqueira Melo et al. 2015). A Syrah grape pomace anthocyanin-rich extract (acidified methanol, pH = 4.0) presented 6.79 mM of Trolox per gram of solid wastes (IC50 = 372 ng/mL) (Trikas et al. 2016). Merlot grape pomace extracts presented the highest antioxidant capacity with 75% acetone and 50% AcN in samples, with average antioxidant activity levels of 77 g of ascorbic acid (AA) equivalents per kg of pomace DW (gAAeq/kg DW) determined by ABTS assay (Ferri et al. 2020). Grape pomace ethanol/water extracts from Portuguese grape varieties presented high antioxidant capacity determined by ORAC (906–2337 μmol TE/g residue) and chelating capacity (55–104% inhibition/mg residue) (Tournour et al. 2015). A hydroalcoholic extract of a Merlot grape pomace maintained close to normal levels several oxidative stress indicators in the plasma, liver and brain in arthritic rats (Gonçalves et al. 2017). Malbec grape pomace extract antioxidant activity determined by ORAC assay resulted in 2756 μmol TE/g extract (Antoniolli et al. 2015). Aqueous grape pomace extract obtained by ultrasound-assisted extraction presented higher phenolic content and antioxidant capacity when compared to conventional extraction and with increasing temperature (González-Centeno et al. 2015). Cabernet Sauvignon grape pomace flour presented 41.11 ± 3.01 mg GAE/g of total polyphenols, 1.49 ± 0.18 mg/g cyanidin 3-glucoside equivalents of total anthocyanin, 362.9 μmol TE/g determined by ORAC (Urquiaga et al. 2018). Red grape pomace extracts obtained by enzymatic-assisted extraction and by high hydrostatic pressure (HHP) resulted in a higher release of polyphenols (higher total polyphenols content) for the combined extraction (enzymatic complex and HPP at 200 MPa from 5 to 10 min), as well as a high proanthocyanidin extraction (Cascaes Teles et al. 2021). Grape pomace antioxidant compounds (polyphenols) have also been extracted by

supercritical fluids finding catechin, procyanidin B2, epicatechin, procyanidin gallate dimer, quercetin glucuronide and syringetin glucoside and the same seven anthocyanins as the most abundant polyphenols found in Cannonau and Cabernet (Floris et al. 2010). Red grape pomace extracts from Merlot, Cabernet Sauvignon, Syrah, Petit Verdot, Tempranillo and Tintilla, obtained by two high pressure extraction techniques, supercritical fluid extraction (CO_2 + 20% ethanol) and pressurized liquid extraction (either ethanol, water or an ethanol/water mixture as the extraction solvents), demonstrated being promising techniques for the green extraction of antioxidant phenolic compounds from red grape pomace (Otero-Pareja et al. 2015).

Proanthocyanidins from grape seed extract has been found to protect antioxidant defenses, specifically, glutathione (GSH), when tested in rat primary glial cell cultures treated with LPS/IFN-γ as well as showing better tolerance against treatment with hydrogen peroxide (H_2O_2) and tert-butyl hydroperoxide when pre-treated with grape seed extract. Primary glial cells play a dual role in neuropathological processes implicating the production of nitric oxide and other radicals including their metabolites and in parallel, they produce GSH to protect other cells from oxidative stress (Roychowdhury et al. 2001). Grape seeds also contain oil with high levels of unsaturated fatty acids (90%), including linoleic (C18:2) and oleic (C18:1), as well as traces of linolenic (C18:3) and palmitoleic (C16:1). These healthy fatty acids could be extracted by supercritical fluid extraction, which represents a green alternative for grape seed oil extraction as well as to preserve its natural phytochemicals (such as antioxidant tocopherols), and has proved antioxidant capacity by DPPH (Passos et al. 2010; Prado et al. 2012). Besides, supercritical fluid extraction of grape seeds has shown to not only extract fatty acids but to also extract natural antioxidants from grape seeds such as catechin, epicatechin, gallic acid and resveratrol, where the extract was enriched in antioxidants in more than 150% concerning the starting extracts by supercritical fluid extraction (Marqués et al. 2013).

In a study conducted on two red wine grape cultivars, Pusa Navarang and Merlot, the first one showed a total phenolic content of 95.8 mg/mL, flavonoids of 30.5 mg/mL and flavan-3-ols of 21.8 mg/mL in seeds extract and its skin extract showed a total anthocyanin content of 4.9 mg/mL. Seed extract showed a better antioxidant capacity. Particularly, skin extract from Pusa Navarang showed the highest total polyphenols and anthocyanins content as well as the highest antioxidant capacity determined by ABTS, DPPH and FRAP. The study showed a correlation between antioxidant capacity and polyphenols, flavonoids and flavan-3-ols content (Doshi et al. 2015). In the case of Tannat grape skin, Fernández-Fernández et al. (2019) found an antioxidant capacity of 29.325 ± 0.897 mg/mL of dry sample by ABTS and 0.150 ± 0.011 μmol TE/mg of dry sample by ORAC-FL, while Tannat grape skin extracts presented higher antioxidant capacity by ABTS and ORAC-FL methods: hydro-alcoholic-acid (0.474 ± 0.036 mg/mL and 0.715 ± 0.063 μmol TE/mg), ethanolic (1.278 ± 0.093 mg/mL and 0.721 ± 0.077 μmol TE/mg) and ultrasound-assisted extracts (0.866 ± 0.047 mg/mL and 0.652 ± 0.031 μmol TE/mg). Hydro-alcoholic-acid extract from Tannat grape skin presented the highest antioxidant capacity which corresponds with higher total polyphenols and total monomeric anthocyanins, presenting great potential as a natural source of antioxidants useful

for ROS neutralization. Deng et al. (2011) reported a total phenolic content of red wine grape peel was of 21.4–26.7 mg GAE/g dry matter and DPPH radical scavenging activity of 32.2–40.2 mg AAE/g dry matter. Total flavanol resulted in 31.0–61.2 mg CE/g dry matter and proanthocyanidin contents were of 8.0–24.1 mg/g dry matter for five wine grape pomace varieties (two white and three red). Fermented Petit Verdot grape skin (after separation of the must in the first step of fermentation) was found to possess higher total polyphenol content (185.53 ± 14.73 μg/mg DW), antioxidant capacity by DPPH (EC50 = 1.10 ± 0.14 μg of ext./μg of DPPH), a vasorelaxant-effect on small rat mesentery artery and a significant reduction on ROS production in small mesenteric artery rings when compared to basal (Albuquerque et al. 2017).

11.3.2 Anti-inflammatory

Chronic inflammation is related to many body complications that may lead to chronic diseases. As previously stated, an over-accumulation of lipids in the adipose tissue might result in the production of pro-inflammatory cytokines, nitric oxide (NO), ROS, and the up-regulation of the expression of transcription factors such as NF-κB. Moreover, other diseases involving gut inflammation such as diarrhea, irritable bowel syndrome, chronic inflammatory bowel disease and other immune-related disorders, and junction inflammation such as arthritis rheumatoid, may be prevented and/or treated by ameliorating inflammation (Chacón et al. 2009). Also, inflammatory and cytotoxic factors could cause neuronal damage in the central nervous system (Jeong et al. 2013). Inflammation is associated with several neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and multiple sclerosis (Glass et al. 2010). Chronic inflammation is also associated with obesity, diabetes and insulin resistance states, where pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-a), interleukin 6 (IL-6), C reactive protein (CRP) and monocyte chemoattracting protein 1 (MCP-1) are secreted as well as displaying deregulation of the levels of the adipokines such as adiponectin and leptin (Chacón et al. 2009). Moreover, NF-κB pro-inflammatory transcription factor regulates the gene expression of IL-2, IL-6, IL-8, IL-1b, and T-cell surface receptors and its activation can interfere with insulin signaling (Chacón et al. 2009). Thus, the search for anti-inflammatory natural sources such as grape byproducts (Fig. 11.1) is of great interest in order to alleviate inflammation consequences.

Taking this interest into account, a lyophilized wine extract obtained from Jacquez grapes showed a decrease of IL-1β-induced nitric oxide production in a dose-dependent manner in human articular chondrocytes (Panico et al. 2006). Polysaccharides from Cabernet Franc, Cabernet Sauvignon, and Sauvignon Blanc wines have shown in vitro anti-inflammatory properties by decreasing NO production and inflammatory cytokines (TNF-α and IL-1β) (de Lacerda Bezerra et al. 2018).

Red and white grape pomace have shown to suppress chronic inflammation induced by lipopolysaccharide (LPS) and galactosamine (GalN) in Sprague–Dawley rats, when orally administered methanolic extracts. The extracts inhibited the activation of NF-κB by LPS/GalN stimulation in a dose-dependent manner, and red grape pomace effect was stronger than white grape pomace. In addition, rats fed an AIN93 M-based diet and red grape pomace-supplemented (5%) for 7 days, was found to suppress the LPS/GalN-induced activation of NF-κB and to inhibit the expression of iNOS and COX-2 proteins. Thus, red grape pomace may have anti-inflammatory potential (Nishiumi et al. 2012). Merlot grape pomace treatment of adjuvant-induced arthritic rats showed to delay the development of the paw edema as well as diminishing the infiltration of polymorphonuclear leukocytes (neutrophils) in the femoro-tibial joint cavities of the legs (Gonçalves et al. 2017). An ethanolic extract from Petit Verdot red grape pomace was found to reduce paw edema and neutrophil migration when compared with control groups as well as reducing TNF-α and IL1-β levels in the peritoneal fluid, representing an interesting source of anti-inflammatory bioactive compounds (Denny et al. 2014). Red wine grape pomace has been reported for preventing the increase of TNF-α and IL-10 levels in a murine model of lethal ischemic heart disease (atherogenic diet-fed SR-B1 KO/ApoER61h/h mice), which is an inflammatory condition, after 7 days of 20% of grape pomace flour intake (Rivera et al. 2019). Red grape pomace extract rich in anthocyanins exhibited anti-inflammatory activity against COX-1 and COX-2 (Trikas et al. 2016). Petit Verdot pomace suppressed TNF-α liberation at the concentration of 10 μg/mL in LPS-induced RAW264.7 macrophages (Siqueira Melo et al. 2015). Tannase-biotransformed grape pomace extracts were found to reduce ROS formation in Caco-2 cells before and after biotransformation at 100 and 200 μg/mL (dry extract w/v), being more potent after biotransformation in the amelioration of inflammation induced by IL-1β in Caco-2 cells, finding great potential as a functional ingredient with anti-inflammatory activity (Martins et al. 2020).

It has been studied the anti-inflammatory potential of procyanidins from grape-seed extract on human adipocytes (SGBS) and macrophage-like (THP-1) cell lines showing a reduction of IL-6 and MCP-1 expression after an inflammatory stimulus when pre-treated with grape seed procyanidin extract. In addition, grape seed procyanidin extract stimuli alone demonstrated to modulate the gene expression of adipokines (APM1 and LEP) and cytokines (IL-6 and MCP-1) as well as partially inhibit NF-κB translocation to the nucleus (Chacón et al. 2009). Grape seed extract supplementation in IL-10-deficient mice (model for human Crohn's disease), showed to down-regulate NF-κB signaling and reduce the expression of TNF-α and IFN-γ (Unusan 2020). Proanthocyanidins from grape seed extract exert no effect on LPS/IFN-γ-induced NO production or iNOS expression but enhance low-level NO intracellular production by primary rat astroglial cultures, as well as protecting GSH pool in microglial cells during high output NO production and better tolerance against H_2O_2 in astroglial cells when pre-treated (Roychowdhury et al. 2001). Furthermore, procyanidins from wild grape (Vitis amurensis) seeds significantly reduced the production of NO, PGE2, and ROS as well as inhibiting pro-inflammatory mediators' protein expression (iNOS and COX-2) and

pro-inflammatory cytokines (TNF-α and IL-1β) in LPS-Induced RAW 264.7 macrophages. Moreover, procyanidins extract prevented nuclear translocation of NF-κB by diminishing inhibitory IκBα, NF-κB and MAPK phosphorylation, representing a potent anti-inflammatory (Bak et al. 2013).

Anthocyanins have shown anti-inflammatory activity as well in murine BV2 microglial cells by inhibiting LPS-induced pro-inflammatory mediators (nitric oxide and prostaglandin E2), pro-inflammatory cytokines (TNF-α and IL-1β), without significant cytotoxicity, and inhibiting the nuclear translocation of NF-κB by diminishing the degradation of NF-κB inhibitor and the phosphorylation of cellular proteins (extracellular signal-regulated kinase, c-Jun N-terminal kinase, p38 mitogen-activated protein kinase, and Akt). Also, anthocyanins downregulated the excessive expression of iNOS, COX-2, TNF-α, and IL-1β in BV2 cells stimulated with LPS (Jeong et al. 2013).

Specifically, Tannat grape skin extract (hydro-alcoholic-acid extract) has shown to reduce LPS-induced nitric oxide production on RAW264.7 macrophages when pre-treated with the extract for 24 h (prevention assay) as well as pre-treated and treated with the extract for 24 h, without displaying cytotoxic effects (Fernández-Fernández et al. 2019). In the same study, cyanidin chloride (5, 10, and 20 μg/mL) anti-inflammatory effect was reported implying this compound is the main responsible for Tannat grape skin extract anti-inflammatory activity (Fernández-Fernández et al. 2019).

11.3.3 Anti-diabetic

Type 2 diabetes is a non-communicable chronic disease that is characterized by prolonged hyperglycemia, where insulin secretion can be partially or completely inhibited, or insulin resistance. Long-term high level of blood glucose causes chronic complications, which involves microvascular lesions that can cause diabetic nephropathy, diabetic retinopathy, and diabetic neuropathy, as well as macrovascular complications which include cardiovascular and cerebrovascular diseases (Xu et al. 2018). Type 2 diabetes is also related to Alzheimer's disease which shows similar symptoms, including insulin resistance and impaired glucose metabolism, causing an insulin transport reduction to the brain with the subsequent aberrant activation of protein kinases in the insulin signaling pathway (PI3K and ERK). When glucose metabolism in the brain is abnormal, glucose autoxidation and advances glycation end products (AGEs) formation increases, which were found to be related to amyloidogenesis. AGEs interaction with its receptor (RAGE) promotes ROS production leading to oxidative stress with the subsequent degradation of the oxidative stress cellular sensor, erythroid 2-related factor 2 (Nrf2). Insulin resistance can cause the hyperphosphorylation of tau, which is a neurodegenerative disease marker such as Alzheimer's disease, and the antioxidant system downregulation leads to neurodegenerative disorders (Liao et al. 2017). Many antidiabetic drugs

(biguanides, sulfonylureas, meglitinides, thiazolidinediones, α-glucosidase and dipeptidyl peptidase-IV inhibitors, incretin mimetics, and insulin) present serious side/adverse effects (Arulselvan et al. 2014), making the search for antidiabetic natural sources of great importance such as red grape by products (Fig. 11.1).

Chronic eye diseases such as cataracts, age-related macular degeneration, diabetic retinopathy and glaucoma have become the leading cause of irreversible vision loss in the elderly population and prevention is vital because of treatment infectiveness. These chronic eye diseases are caused by oxidative stress and chronic inflammation. In the case of cataracts, the disruption of the lens protein architecture is the cause of blindness which can be caused by oxidative stress-inducing lens protein aggregation, thus flavonoids have great potential for common cataract and diabetic cataract prevention. Some grape skin flavonoids such as myricetin, cyanidin, rutin, among others, have been found to potentially inhibit diabetes-induced cataracts. Moreover, polyphenols have been related to the inhibition effects of age-related macular degeneration (AMD), more precisely, anthocyanins have been found to help reverse oxidative stress and have an ocular protective effect. Diabetic retinopathy is a microvascular complication that involves the breakdown of the blood-retinal barrier which in early stages swelling of blood vessels cause leaks and edema. Prolonged hyperglycemia induces oxidative stress by the formation of advanced glycation end products (AGEs) and cellular inflammation, leading to the damage of the retina. Polyphenols have been found to help in the prevention and/or retardation of diabetic retinopathy progression. Glaucoma is a progressive neurodegeneration that involves oxidative stress, inflammation, mitochondrial dysfunction, glial cell dysfunction and activation of apoptotic pathways. Polyphenols have been found to ameliorate the damage by positively influence inner retinal functional. Specifically, grape polyphenols have been found to reduce the expression of inflammatory cytokines and the accumulation of leukocytes in eyes and retinal leakage in C57BL/6 mice (Xu et al. 2017). An anthocyanin extract from blueberry showed protective effects against oxidative injuries induced by H_2O_2 in human retinal pigment epithelial cells through decreasing ROS and malondialdehyde levels and increasing superoxide dismutase, catalase, and glutathione peroxidase levels. Anthocyanins activated Akt-signal pathways and decreased vascular-endothelial-cell-growth-factor levels. Thus, blueberry anthocyanins could prevent and stop the progression of age-related macular degeneration by antioxidant mechanisms (Huang et al. 2018).

Risk factors comprise overweight/obesity, physical inactivity and an unhealthy diet. An important strategy for prevention and/or treatment of type 2 diabetes is the inhibition or retardation of enzymatic activities of the carbohydrases α-amylase (Sun et al. 2019) and α-glucosidase (Fernández-Fernández et al. 2019), in order to avoid post-prandial blood glucose pick, and the inhibition of advanced glycation end products (AGEs) formation (Bastos and Gugliucci 2015). Another strategy is the inhibition of glucose transporters such as GLUT-2, GLUT-4, and SGLT-1 at the level of the intestinal cell (Wang et al. 2018a). Also, the improvement of insulin receptors sensitivity by the enhancement of Akt/PI3K pathway contributes to diminish insulin resistance and to promote glucose transporters translocation to cells lipid bilayers as well as the up-regulation of glucose transporters expression. Also, type

2 diabetes involves a pro-inflammatory state as well as an overproduction of ROS which influences insulin sensitivity through insulin receptor phosphorylation and ectopic fat deposits related to the development of obesity-related cardiovascular diseases. These strategies can be approached by natural bioactive compounds such as polyphenols (Arulselvan et al. 2014; Hatia et al. 2014) with the potential of delaying diabetic complications and altering metabolic abnormalities through cellular and molecular mechanisms (Arulselvan et al. 2014). Dietary antioxidants that inhibit peroxidation chain reactions have been associated with type II diabetes risk reduction, regulating weight control, and blood glucose in diabetic patients (Doshi et al. 2015).

Intestinal glucose absorption is carried out by sodium-dependent glucose transporter-1 (SGLT1) and glucose transporter 2 (GLUT2). Free glucose concentration in the intestine lumen varies depending on the meal: before the meal, the concentration is <5 mM (low) and SGLT1 in the apical side of the enterocyte actively transports available glucose into the intestinal cell, GLUT2 in the basolateral membrane is also active postprandial to maintain cellular metabolism transporting glucose from the blood into the cell, and during the meal glucose concentration starts increasing (5–10 mM) and is transported by SGLT1 from the intestinal lumen and subsequently into the systemic circulation via GLUT2 (Wang et al. 2018a). After the meal, very high glucose concentrations (25–100 mM) are detected because of food carbohydrates hydrolysis by α-glucosidase located on the apical enterocyte membrane, producing monosaccharides that are absorbed by SGLT1 and GLUT2 in the apical side. There has been evidence of inhibition of GLUT2 by flavonoids such as quercetin and myricetin (Wang et al. 2018a) that are present in red grape pomace. Anthocyanins have shown several antidiabetic activities. These may decrease glucose levels, activate insulin receptor phosphorylation, increase GLUT-4 expression, and prevent pancreatic apoptosis in STZ-induced diabetic rats. They might also activate AMPK, up-regulate GLUT4 to improve insulin sensitivity, suppress glucose production and inactivate acetyl-CoA carboxylase in T2DM mice. Also, anthocyanins could enhance the secretion of adipokine (adiponectin and leptin), as well as increasing the mRNA levels of PPARγ in isolated rat adipocytes. Among anthocyanins, cyanidin 3-glucoside has shown to ameliorate insulin sensitivity and hyperglycemia, up-regulate GLUT-4 levels, reduce the levels of fasting glucose, and to reduce the secretion of inflammatory cytokines via JNK/FoxO1 signaling pathway (Xu et al. 2018). Anthocyanins from purple corn have shown antidiabetic (insulin secretion activity, anti-hyperglycemic activity, and HbA1c-decreasing activity) and beta cell-protection activities from cell death in HIT-T15 cell culture (pancreatic beta cell culture) and db/db mice (Hong et al. 2013). Proanthocyanidins have been reported for inhibiting digestive enzymes such as α-amylase and α-glucosidase, decreasing hyperinsulinemia (enhance adiponectin secretion in white adipocytes and promote GLUT-4 expression in skeletal muscle), reducing postprandial glycemia, improving insulin sensitivity, inhibiting insulin and β-cell mass secretion, and reducing anti-inflammatory activity (Unusan 2020).

Bioactive polysaccharides have shown to restore the body and fat mass weight, improve glucose tolerance ability, reduce fasting blood glucose levels, increase

hepatic glycogen level, ameliorate insulin resistance, increase HDL-C levels and decrease TC, TG and LDL-C levels, when tested on a high-fat diet and STZ-induced type 2 diabetic mice compared to the control diabetic mice. It also showed to alleviate lesioned organ tissues such as liver, kidney, and pancreas, and to be involved in activating PI3K and Akt phosphorylation as well as the translocation of GLUT4 in diabetic mice (Wang et al. 2017b).

Pusa Navarang and Merlot grapes by-products (seeds, skin and berry stems) have shown to stimulate insulin secretion on mice pancreatic islets at basal glucose level (5.5 mM) and at enhanced glucose level (16.5 mM) compared to mice without extracts supplementation (insulinotropic effects). Pusa Navarang grape skin and stems presented the highest insulin stimulation in both conditions with a better stimulation of secretion by the berry stems' extract. In the case of grape skin, anthocyanins could be responsible for the insulinotropic effect. Grape pomace extracts exert an anti-postprandial hyperglycemic effect, which could be a source of antioxidants and anti-hyperglycemic compounds to regulate blood glucose levels and oxidative stress associated with Type 2 diabetes (Doshi et al. 2015). Red wine grape pomace flour has shown to improve fasting glucose and postprandial insulin levels in a randomized controlled trial of 16-week conducted on 38 human males (30–65 years of age) with at least one component of metabolic syndrome (Urquiaga et al. 2015).

Altered glucose metabolism in the brain is associated with cognitive decline, and powder from Taiwan grapes was found to reduce RAGE expression and tau hyperphosphorylation in the brain tissues of aged Wistar rats fed with high-fructose–high-fat diet and 6% of grape powder. In contrast, grape powder upregulated the expression of Nrf2 and BDNF, and the phosphorylation of PI3K and ERK. Thus, grape powder demonstrated the potential to ameliorate changes in proteins associated with neurodegeneration in the brain of aged rats fed a high-fructose–high-fat diet (Liao et al. 2017).

Red grape pomace extracts obtained by enzymatic-assisted extraction and by high hydrostatic pressure (HHP) were found to inhibit α-amylase in a 92.31% (IC50 = 0.054 g/mL) for the combined extraction (enzymatic complex and HPP at 200 MPa from 5 to 10 min) (Cascaes Teles et al. 2021). Tannat grape skin has demonstrated α-glucosidase inhibition capacity for several extracts. IC50 values for α-glucosidase inhibition capacity were reported for hydro-alcoholic-acid, ethanolic and ultrasound-assisted extractions (888.5 ± 79.3, 2584.1 ± 211.1, and 1966.1 ± 109.4 μg/mL, respectively), compared to acarbose, chlorogenic acid, and cyanidin chloride standard (4.0 ± 0.3 μg/mL, 69.1 ± 1.6 μg/mL, and 95.5 ± 1.8 μg/mL, respectively). Hydro-alcoholic-acid Tannat grape skin extract rich in anthocyanins, showed the best inhibition capacity among the extracts, suggesting its potential in helping with the regulation of type II diabetes by retarding post-prandial blood glucose increase (Fernández-Fernández et al. 2019). Berry extracts from black currant and rowanberry showed inhibition of α-glucosidase with IC50 values of 20 and 30 μg GAE/mL respectively, being as effective as acarbose (pharmaceutical inhibitor) (Boath et al. 2012).

11.3.4 Anti-obesity

The abnormal or excessive accumulation of fat in the body is called overweight and obesity, respectively, which are associated with the development of metabolic diseases such as metabolic syndrome, hypertension, cardiovascular diseases and type 2 diabetes (Hatia et al. 2014). At the cellular level, obesity characterizes by an augment in the number (hyperplasia) and size of adipocytes (hypertrophy). Adipocytes TAG synthesis may be a body mechanism to counteract the large number of other molecules present in the blood, mainly fat and glucose, implying a risk increment of hyperlipidemia and hyperglycemia due to its inhibition, to lipotoxicity and glucotoxicity, respectively. Lipotoxicity is associated with diabetes pathogenesis as a consequence of lipid overloaded pancreatic β-cells leading to a reduction in β-cell mass (Torabi and DiMarco 2016).

Higher consumption of nutrients (carbohydrates and fat) than needed leads to overweight and obesity. Thus, the inhibition of fat absorption involving pancreatic lipase enzyme may prevent/treat obesity. Accumulation of fat induces an augment on preadipocytes' proliferation and differentiation into adipocytes. In consequence, adipocytes present intracellular dysfunction involving endoplasmic reticulum and mitochondrial stress. Free fatty acids cause reticulum stress leading to oxidative stress in the mitochondria, generating an imbalance on reactive oxygen species (ROS). Cell overproduction of ROS in adipocytes induce insulin resistance, contributing to diabetes caused by obesity. ROS reduce antioxidant endogenous system leading to the damage of free radicals and peroxides on biomolecules (DNA, lipids, and proteins) as well as the dysregulation of adipokine secretion. Consequently, it causes the production of pro-inflammatory cytokines (interleukin-6, tumor necrosis factor-α, monocyte chemoattractant protein-1) and the reduction in anti-inflammatory molecules (adiponectin) by preadipocytes, macrophages and adipose stem cells. Adipose tissue chronic inflammation caused by cytokines secretion seems to play an important role in desensitizing cells to insulin (Hatia et al. 2014). Thus, obesity is related to oxidative stress and inflammation (Gerardi et al. 2020).

Polyphenols, particularly proanthocyanidins (condensed type tannin bonded by condensation or polymerization such as flavan-3-ols and flavan-3,4-diols) have been found to inhibit pancreatic lipase (Shihui Wang et al. 2014). Polyphenols (epicatechin gallate, chlorogenic acid, 3,4-dihydroxy-benzaldehyde, naringenin, quercetin and the microbial metabolite 3,4-dihydroxyphenylacetic acid) have shown to reverse the detrimental effect of H_2O_2 and cytotoxicity on 3T3-L1 preadipocytes. Besides, epicatechin gallate, epicatechin, genistein, naringenin, curcumin and 3,4-dihydroxyphenylacetic acid reduced basal IL-6 secretion as well as H_2O_2 co-exposition (Hatia et al. 2014). Proanthocyanidins stimulate glucagon-like peptide 1 (GLP-1)/dipeptidylpeptidase 4 (DPP4) activity that inhibits the neuropeptides associated with food consumption and satiety, as well as inhibiting fat absorption through lipase inhibition, reducing adipocyte hypertrophy, decreasing hyperinsulinemia (enhance adiponectin secretion in white adipocytes and promote GLUT-4

expression in skeletal muscle), inhibiting insulin and β-cell mass secretion, and decreasing obesity-mediated chronic inflammation (Unusan 2020).

In another study conducted on 3T3-L1 and 3T3-F442A fibroblasts or preadipocytes (common in vitro models for studying adipocyte differentiation), authors showed that grape pomace extracted polyphenols upregulated protein level of glucose transport protein 4 (GLUT4), p-PKB/Akt, and p-AMPK in 3T3-F442A. Adipocytes also showed increased mRNA expression of fatty acid synthase, lipoprotein lipase, adiponectin, GLUT4, and peroxisome proliferator-activated receptor γ, while it decreased mRNA expression of leptin and Insig-1. The authors stated grape pomace extracted polyphenols may induce adipocyte differentiation by the upregulation of adipogenic genes, GLUT4, and PI3K (Torabi and DiMarco 2016). Red wine pomace (*Vitis vinifera L.* cv. Tempranillo) intake in Wistar rats (100 mg/kg body weight) was found to reduce body weight, abdominal fat area, liver weight and lipids deposition with increased antioxidant status, blood glucose levels, adipocyte size and increased Lactobacillus spp./Bacteroides spp. ratio (Gerardi et al. 2020).

In particular, Tannat grape skin has shown pancreatic lipase inhibition capacity for hydro-alcoholic-acid (IC50 = 2431.0 ± 79.9 μg/mL), ethanolic and ultrasound-assisted extracts, compared to chlorogenic acid, cyanidin chloride, gallic acid, caffeine, and rutin IC50 values (11.9 ± 1.4 μg/mL, 56.9 ± 6.6 μg/mL, 332.5 ± 32.1 μg/mL, 241.1 ± 0.8 μg/mL, and 290.0 ± 20.6 μg/mL, respectively). The hydro-alcoholic-acid extract showed the best pancreatic lipase inhibition capacity of the studied extracts, suggesting a great potential for obesity treatment (Fernández-Fernández et al. 2019).

11.3.5 Cardiovascular Health Properties

Cardiovascular diseases are associated with modified fatty acid metabolism and LDL's excessive lipid peroxidation, which implicates the formation of thromboxane, leading to enhanced platelet aggregation, with the consequent artery blockage and thrombosis (Yu and Ahmedna 2013). The accumulation of lipid oxidation products from LDL could be prevented by the presence of plasma antioxidants. ROS overproduction is associated to several disorders such as hypertension, which comprises an augment on superoxide anion and hydrogen peroxide formation, the reduction of nitric oxide synthesis, as well as decreased antioxidant bioavailability (Albuquerque et al. 2017). It is known that high concentrations of serum cholesterol, in particular LDL-cholesterol, represents a risk factor for atherosclerosis (accumulation of cholesterol deposits at the arterial wall that triggers an inflammatory response which contributes to the development of ischemic cardiovascular disease) and coronary heart disease, which can be reduced by lowering plasma lipid concentrations, especially LDL, and by increasing plasma HDL concentrations (protective against coronary heart disease) (Martín-Carrón et al. 2000; Rivera et al.

2019). Some polyphenols are known to reduce the absorption of cholesterol at the intestine of rats by diminishing the solubility of cholesterol in micelles. Consequently, bile acids return to the liver is lowered, leading to an augment on their hepatic synthesis from cholesterol, which causes a higher expression of hepatic LDL receptors that involves a reduction of LDL lipoproteins and serum cholesterol (Martín-Carrón et al. 2000). Fruit and fiber increase intake is associated with a risk reduction of cardiovascular disease (Zhu et al. 2015). In addition, soluble dietary fiber possesses hypocholesterolemic activity by forming gels in the gastrointestinal tract that decrease the absorption of cholesterol in the intestinal lumen (Pérez-Chabela and Hernández-Alcántara 2018).

Red wine grape pomace has been reported for attenuating atherosclerosis and myocardial damage in a murine model of lethal ischemic heart disease (atherogenic diet-fed SR-B1 KO/ApoER61h/h mice fed with 20% of grape pomace flour), as well as to increase the survival by improving plasma antioxidant activity (increased HDL-containing plasma antioxidant activity) and modulating inflammation by decreasing pro-inflammatory cytokine (TNF-α and IL-10) levels, having the potential to decrease the progression of atherosclerosis, reduce coronary heart disease, and improve cardiovascular outcomes (Rivera et al. 2019). Red grape pomace intake (20 g/day) in a 16-week longitudinal intervention study with 38 males (30–65 years of age) significantly decreased systolic and diastolic blood pressure (Urquiaga et al. 2015). Grape products have shown a reduction of atherogenic markers, cardioprotection and reduction of the effects in lipid profile and blood pressure. Moreover, grape pomace intake in Wistar rats have shown to reduce HMG-CoA reductase activity in the liver (the enzyme that participates in cholesterol synthesis) and increase the fractional plasma cholesterol catabolic rate (Zhu et al. 2015). Martín-Carrón et al. (2000) conducted a study on adult Wistar rats, finding that the intake of grape products increased stool weight and the amount of fat and protein excreted in feces as well as lowered serum total cholesterol and LDL cholesterol concentrations in hypercholesterolemic rats. Cholesterol-free diet rats were fed with red grape peel obtaining an increment on HDL-cholesterol compared to rats fed with cellulose, but on changes were found on triglyceride concentrations. On the cholesterol-added diet, rats decreased total cholesterol and LDL-cholesterol concentrations compared to the control group, not finding changes on HDL cholesterol and triglyceride concentrations. Grape skin effect on cholesterol and lipoprotein concentrations could be attributed to fiber characteristics or to the polyphenolic fraction (Martín-Carrón et al. 2000).

Procyanidins have shown to inhibit human endothelial NADPH oxidase, which is responsible for ROS overproduction and resveratrol alone has shown anti-atherogenic and anti-inflammatory effects in hypercholesterolemic-diet rabbits (1% cholesterol) (Yu and Ahmedna 2013). Moreover, the main vasoactive polyphenols in red wine are proanthocyanidins, which induce the endothelium-dependent dilatation of blood vessels and inhibit vasoconstrictive peptide endothelin-1 synthesis (Da Silva et al. 2013). Proanthocyanidins contribute to cardiovascular health by diminishing lipid peroxidation, contributing to lipid homeostasis by enhancing the opposite transport and removal of cholesterol in bile, decreasing plasma triglycerides and

apolipoprotein B, reducing atherosclerotic risk, reducing dyslipidemia, inhibiting lipoprotein secretion, antihypertensive properties (e.g. by delayed endothelial aging), reducing blood pressure, plasma homocysteine concentrations, and serum C-reactive protein (Unusan 2020).

Petit Verdot grape skin extract from pomace, fermented and unfermented (fresh) grape skins were found to elicit vasorelaxation as well as in vitro free radical scavenger activity confirmed by determining ROS production in small mesenteric artery rings of rats. The vasorelaxation induced by fermented grape skin was about 10 times more potent than that induced by the unfermented. The results suggested that the mechanism of action is dependent on endothelium-derivative relaxant factors such as NO and EDHF. ROS formation in treated vessels (fermented and unfermented) was significantly reduced when compared to basal conditions, but fermented grape skin showed a marked antioxidant effect on the tissue (Albuquerque et al. 2017). Anthocyanins seem to have a positive role in preserving cardiovascular health, lowering the risk of myocardial infarction and mortality related to cardiovascular diseases, but the underlying molecular mechanisms of action are not entirely clear (Krga and Milenkovic 2019).

11.3.6 Anti-carcinogenic

Cancer development is associated with abnormal cell cycle progression, abnormal cell proliferation, oxidative stress damage, and inhibition of cancer cells' apoptosis (programmed cell death), acting on intracellular molecular signaling related to the initiation and/or promotion of cancer. Dietary polyphenols may have a positive effect on fighting the onset of cancer by antioxidant properties, protein kinases' inhibition, reduction of protease activities, altering phase-I and phase-II drug-metabolizing enzymes, blocking of receptor-mediated functions, alteration of cell cycle checkpoint controls, transcription factor expression and apoptosis, epigenetic changes in promoter methylation and chromatin remodeling, inhibition of angiogenesis, invasion and metastasis (Yu and Ahmedna 2013). The most important bioactive compounds with anti-cancer activity are flavonoids (Georgiev et al. 2014), such as the ones present in red grape byproduct (Fig. 11.1).

Procyanidins present in grape seeds have shown to exert cytotoxicity on human breast, lung, gastric adenocarcinoma cells while enhancing the growth and viability of gastric mucosal cells. Furthermore, grape seed extract has shown to protect skin from UV-radiation-induced oxidative stress (which may lead to skin cancer) and to activate the signals mediated by mitogen-activated protein kinase and NF-κB in human epidermal keratinocytes. It may also be helpful in the treatment of colorectal cancer by growth inhibitory and apoptosis-inducing effect as well as in the inhibition of MOLT-4 leukemia cell growth. On the other hand, resveratrol has shown to inhibit tumor initiation, promotion and progression as well as enhancing apoptotic effects of cytokines, chemotherapeutic agents and gamma-radiation (Yu and Ahmedna 2013). Proanthocyanidins present antiproliferative and antiangiogenic

effects, induce apoptosis, cell cycle arrest, and inhibit metastatic processes in the lung, liver, pancreas, colorectal, prostate, breast, and skin (Unusan 2020). Lyophilized red grape pomace also shows a chemopreventive effect on spontaneous intestinal tumorigenesis in the ApcMin/+ mouse model and grape powder seems to have a beneficial in the prevention of colon cancer (Zhu et al. 2015). *Vitis vinifera* "Currant" and "Sultana" extracts exhibited anti-cancer activity by the prevention of colon cancer for their antioxidant and anti-inflammatory properties, and grape seed proanthocyanidins reduced cell viability and induced apoptosis in a dose- and time-dependent manner in human pancreatic cancer cells (migration inhibition by inactivation of NF-κB) (Georgiev et al. 2014).

11.3.7 Gut Microbiota Health Improvement

Beneficial gut microbiota (genera Lactobacillus and Bifidobacterium) is associated with health improvements through vitamin synthesis (B-group and K), conversion of non-digestible food components (dietary fiber) into short-chain fatty acids, pathogens degradation, modulation of the immune system of the host, influence on brain development and as a modulator of host behavior ('microbiota-gut-brain-axis'). On the other hand, Clostridium, Eubacterium and Bacteroides involve negative effects on health such as diarrhea, irritable bowel syndrome, chronic inflammatory bowel disease and other immune-related disorders, as a consequence of the disruption or dysbiosis of gut microbiota (Nash et al. 2018). A higher Firmicutes/Bacteroidetes ratio is associated with obese and metabolic syndrome subjects (Espín et al. 2017).

Soluble dietary fiber has shown to reduce body weight gain and excessive accumulation of white fat tissue in a high fat diet-induced obese mouse model, and gut microbiota was characterized by a decreased ratio of Firmicutes/Bacteroidetes (phylum level), and an increased abundance of the genera Roseburia (genus level). Also, it was observed an increase in energy expenditure, but not a change energy intake. Thus, the increment of gut microbiota diversity and the colonization of beneficial bacteria by soluble dietary fiber intake improves energy homeostasis and prevents obesity (Wang et al. 2018b). There is also evidence of polyphenols with prebiotic effect promoting gut health, such as ellagitannins, lignans, isoflavones and flavanones that are substrates for the gut microbiota and may exert health benefits in the gastrointestinal tract by their gut microbiota-derived metabolites involving systemic effects (Espín et al. 2017). Randomized controlled trials have stated a significant modulation of intestinal microbes affecting mainly cardiovascular disease markers by polyphenols as prebiotics, with negative correlations between Bacteroides with triacylglycerides, high-density lipoprotein, diastolic blood pressure, and systolic blood pressure; Lactobacillus and triacylglycerides, C-reactive protein; Bifidobacterium with cholesterol and C-reactive protein (Moorthy et al. 2020). The impact of probiotic supplementation in the microbial metabolism of red grape pomace polyphenols on the Dynamic Gastrointestinal Simulator (simgi®) was

assessed finding that the inclusion of *Lactobacillus plantarum* CLC 17 in the colon compartments leads to the formation of more phenolic metabolites (benzoic acids), which may be because of high-molecular-weight procyanidin polymers breakdown (Gil-Sánchez et al. 2020). Red grape pomace and seed polyphenol extracts from Kyoho grape (*Vitis vinifera* "Kyoho") have been reported for improving the recovery of gut microbiota after antibiotic cocktail treatment in high-fat diet-fed C57BL/6J mice supplemented for 7 days after withdrawal of antibiotics compared to the spontaneous recovery group, as well as changing gut microbiota diversity (changes of Verrucomicrobia and Akkermansia in feces) (Lu et al. 2019).

Among grape polyphenols, particularly proanthocyanidins present in grape seeds, have shown to reach the colon via the small intestine (initial site for glucuronidation), where small amounts are absorbed, thus the microbiota form metabolites including benzoic acid, 2-phenylacetic acid, 2-(3′-hydroxypenyl) acetic acid, 3-(3′-hydroxyphenyl) propionic acid, 3-phenylpropionic acid, 2-(4′-hydro- xyphenyl) acetic acid, and hydroxyphenylvaleric acid, increasing bacteria such as Bifidobacterium and Lactobacillus spp. (Unusan 2020). A source of anthocyanins (cranberry) was studied for its impact on gut health (Rodríguez-Morató et al. 2018). Cranberry intake for 5 days in 11 healthy adults was found to attenuate animal-based diet-induced changes in microbiota composition and functionality. The study characteristics were: randomized, double-blind, cross-over, control group was given an animal-based diet plus 30 g/day placebo powder and the rest was given a cranberry diet which included an animal-based diet plus 30 g/day freeze-dried whole cranberry powder (Rodríguez-Morató et al. 2018). The control diet implied 46 taxonomic clades modifications taking pre-diet into account, with the characteristic of Firmicutes increase and Bacteroidetes decrease, compared to cranberry diet that 9 taxonomic clades were modified, showing the opposite tendency for Firmicutes and Bacteroidetes, increasing secondary bile acids, urinary anthocyanins, and bacterially derived phenolic acids by contrast short-chain fatty acids decrease (Rodríguez-Morató et al. 2018).

11.3.8 Anti-bacterial Activity

Among polyphenols properties, defense against biotic stress relates with antimicrobial, anti-fungal, and anti-herbivore properties (Da Silva et al. 2013). Grape pomace extracts have been recognized by their antibacterial capacity against *Bacillus cereus*, *Staphylococcus aureus*, *Campylobacter coli*, *Escherichia coli* O157:H7, *Salmonella infantis*, and *Listeria monocytogenes* ATCC 7644, as well as showing bactericidal effects against total aerobic mesophilic bacteria, lactic-acid and Enterobacteriaceae (Beres et al. 2017). A study conducted on three hydro-ethanolic extracts from Merlot grape pomace showed higher antibacterial activity for Gram-positive bacteria than for Gram-negative bacteria, exhibiting highest inhibitory activities against *Enterococcus faecalis* and *Listeria monocytogenes*, being compromised the activity by simulated in vitro digestion (2-fold reduction) and simulated colonic

fermentation (Corrêa et al. 2017). Syrah red grape pomace and wine extracts exhibited a dose-dependent antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Bacillus cereus* that cause several human infections, being more effective against E. coli and S. aureus (Trikas et al. 2016). Pinot Noir and Merlot wine grape pomace extracts showed antibacterial activity against *Listeria innocua* ATCC 51142 and *Escherichia coli* ATCC 25922, being lower for the latter, as well as the antibacterial activity against both E. coli and L. innocua displayed by films based on Merlot wine grape pomace extract (Zhu et al. 2015). Among grape seeds polyphenols, proanthocyanidins have shown to inhibit bacterial adhesion and coaggregation, with the concomitant reduction of biofilm formation and decreased inflammation: *Bacillus cereus*, *Bacillus coagulans*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, Micrococcus luteus, S. aureus, *Listeria monocytogenes* (Unusan 2020).

11.4 Bioaccessibility and Bioavailability of Bioactive Compounds of Grape By-products: Simulation of Digestion, Cell Studies and In Vivo Studies

The biological activity of bioactive compounds such as polyphenols is subjected to their bioaccessibility and bioavailability. Bioaccessibility implies the release of polyphenols from the food matrix during digestion to be absorbed and metabolized to exert their health-promoting effects (Pešić et al. 2019). Bioaccessibility of grape pomace polyphenols is related to the proportion of non-extractable polyphenols, which are bound to grape fiber, and the portion of the intestine. Furthermore, non-extractable polyphenols are not bioaccessible in the small intestine but they can be at least partially released by large intestinal microbiota from fiber matrix (Yu and Ahmedna 2013). The main reason for bioaccessibility impairment of grape polyphenols is their tightly bound to cellulose and pectin, thus enzymatic hydrolysis could enhance the release of monomeric and oligomeric polyphenolic compounds from their conjugates, facilitating their upper gastrointestinal tract absorption (Martins et al. 2016). Thus, grape pomace fiber may be used as polyphenols carriers to be destined for large intestine (Yu and Ahmedna 2013), or polyphenols may be extracted from grape by-products matrix by ultrasound-assisted extraction, acidic extraction (Fernández-Fernández et al. 2019; González-Centeno et al. 2014), supercritical fluids extraction (Da Porto et al. 2015; Yilmaz et al. 2011), biotransformation by using enzymes (e.g., pectinases, cellulases, and glucanases) (Albuquerque et al. 2017; Martins et al. 2016). Another important reason is the instability and/or degradation of polyphenols during digestion (Pešić et al. 2019). Figure 11.2 exemplifies the steps for polyphenols evaluation of effective bioactivity through bioaccessibility and bioavailability studies. To exert their biological activities, bioactive compounds must be bioavailable, which implies being effectively absorbed from the gut into the circulation and to achieve target tissue. In the case of anthocyanidin

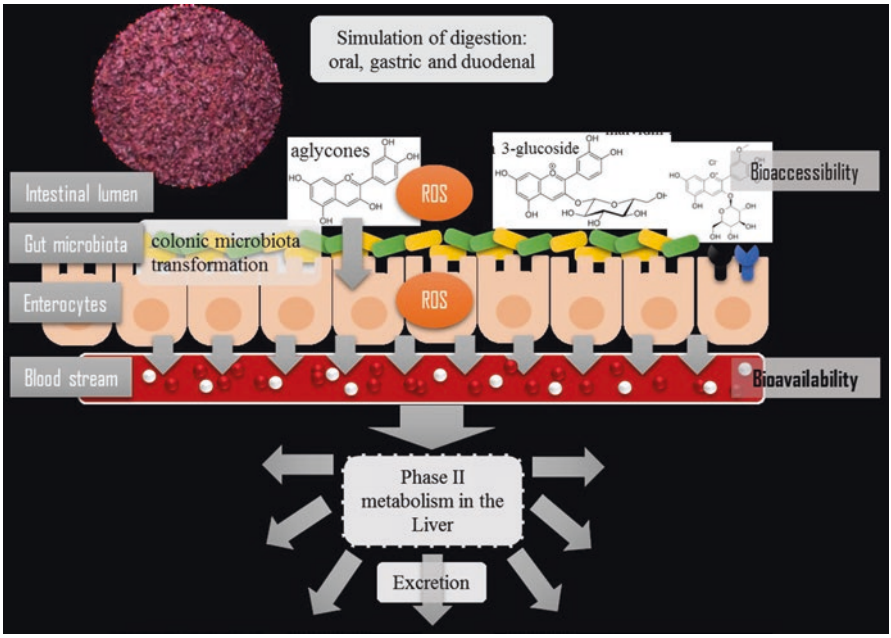

Fig. 11.2 Scheme of red grape pomace bioaccessibility and bioavailability studies, starting with in vitro simulation of digestion to state polyphenols stability/degradation, then small intestine and colon cell lines to study polyphenols bioactivity and absorption ending in the systemic circulation, followed by bioavailability studies including polyphenols metabolism, biodistribution with bioactivity analysis and excretion

aglycones, their absorption is through passive diffusion across the membrane of the gut epithelial cells because of having greater hydrophobicity. In contrast, anthocyanin glycosides are more hydrophilic with high molecular weight, which are supposed to have difficulty in being absorbed in the digestive tract by intestinal microbiota hydrolysis into aglycone form or degradation to phenolic acids. Still, anthocyanins have been reported to have an in vivo role and can be absorbed directly from the stomach (Liang et al. 2012). Even though grape polyphenols present low absorption capacity because only aglycones can effectively pass through the gut wall, they may have a direct positive impact on gut mucosa or can be hydrolyzed by the gut microbiota into aglycones or be degraded (Georgiev et al. 2014). In some food matrixes (mushrooms), polysaccharides bioactivities are affected by digestion conditions, finding negative effects (decreased antioxidant capacity) after intestinal digestion. However, some bioactivities such as α-amylase and α-glucosidase inhibitory activities are significantly increased after digestion meaning bioactive polysaccharides could be a post-prandial hyperglycemia controller (Wang et al. 2018c).

Bioavailability implies the arrival of bioactive compounds into the systemic circulation and their maintenance of availability to be used by cells or tissues. In general, it is considered that polyphenols must be absorbed, metabolized and bioavailable in order to exert their beneficial effects, but in some cases health effects

may be exerted before their absorption through the gut barrier. This is the case of anthocyanins that may protect against the oxidative damage in the gastrointestinal tract which is implicated in degenerative diseases such as colorectal cancer or inflammatory bowel disease. In general, monomeric and low molecular weight polyphenols are absorbed in the upper small intestine in contrast with higher molecular-weight polymers that are first metabolized by colonic microbiota and then absorbed in the large intestine (Pineda-Vadillo et al. 2016). Polyphenols bioavailability and absorption rate into the circulatory system determines their bioactivity as well as the foods with which are consumed. After enzymatic deglycosylation, only 5–10% of ingested dietary polyphenols are absorbed in the small intestine. Mostly, reach the colon intact (90–95%) to be degraded into simpler phenolic acids by gut microbiota, with the consequent absorption into the systemic circulation (Nash et al. 2018). Most polyphenols cannot be absorbed in their native form because of their common form (esters, glycosides, or polymers) usually present in food, being necessary their hydrolysis by endogenous enzymes or microbiota. Their bioavailability is compromised by xenobiotics metabolism, in comparison to micro- and macronutrients (Chedea et al. 2018).

To state polyphenols biological effects, bioaccessibility and bioavailability studies are needed. For this purpose, a global approach including in vitro and in vivo studies is needed. The rate of absorption and bioavailability of bioactive compounds are generally addressed in vivo, which present limitations such as high costs and huge variability (Chedea et al. 2018). However, cell models may represent a suitable alternative for in vivo studies because of its lower cost and bigger screening capacity (Chedea et al. 2018).

Bioaccessibility studies on anthocyanins from pinto beans, black beans and black lentils (delphinidin 3,5-diglucoside, cyanidin 3-glucoside and cyanidin 3,5-diglucoside) showed their absence in the intestinal phase because of pH instability (Giusti et al. 2019). Moreover, cyanidin 3-glucoside degradation to protocatechuic acid has been reported during gastrointestinal digestion (Giusti et al. 2019). In another study, the bioaccessibility of mulberry (Morus atropurpurea Roxb.) anthocyanins showed a decrement after the intestinal digestion, however, the digest showed good antioxidant activity due to anthocyanins degradation with the subsequent generation of phenolics under intestinal conditions (Liang et al. 2012). Polyphenols from wild blueberry (*Vaccinium angustifolium*) have shown high stability (total polyphenols and anthocyanins) during gastric digestion phase (approximately 93% and 99% of recovery, respectively), but decreased during intestinal phase (49% and 15%, respectively) compared to non-digested samples. Also, the complex polyphenol mixture was degraded to a few polyphenols (syringic, cinnamic, caffeic, and protocatechuic acids) during chemostat fermentation that simulates colonic digestion, and after chemostat fermentation acetylated anthocyanins were detected in low amounts. Colonic fermentation might affect blueberry polyphenols bioactivity because of catabolites showing lowered antioxidant activity and cell growth inhibition potential (Correa-Betanzo et al. 2014). Another source of anthocyanins, chokeberry (Aronia melanocarpa) pomace powder, was found to reduce the initial content of total polyphenols by 40% when temperatures up to

140 °C were applied, not altering dietary fiber structure or content, and after in vitro digestion the retained polyphenols were fully bioaccessible, with antioxidant capacity remaining unchanged and with slight reduction of glucose bioaccessibility (Schmid et al. 2020). To improve anthocyanin stability and residence time in the upper digestive tract, which causes a partial absorption (Gadioli Tarone et al. 2020), different blends and/or encapsulation techniques may be used. Polyphenols from blueberry (Vaccinium angustifolium Aiton) and muscadine grape (Vitis rotundifolia) pomaces with a rice-pea protein isolate blend (protein-polyphenol aggregate particles) showed better stability during gastrointestinal transit (in vitro gastrointestinal model) through the protection of polyphenols allowing them to reach gut microbiota and preserve their bioactivity (Xiong et al. 2020). Colloidal carrier systems such as cyclodextrin, polymeric particles, liposomes, and emulsions, could be suitable for encapsulating anthocyanins. Among "top-down" colloidal systems, liposomes represent a promising carrier because of protecting, entrapping hydrophilic bioactive compounds and enabling intestinal absorption, as well as emulsions for protecting from the environment and gastrointestinal conditions, and regarding "bottom-up" colloidal systems spray-drying has been successfully used for anthocyanin protection (Gadioli Tarone et al. 2020). Micro-encapsulation of grape skin anthocyanin-rich extract using emulsification/internal gelation associated with spray/freeze-drying techniques showed favored anthocyanin retention in the microcapsules by spray-drying as well as improving the prolonged release of anthocyanins in simulated gastrointestinal digestion (Zhang et al. 2020).

However, anthocyanins may interact with some of the other food ingredients affecting their bioaccessibility. In a newly developed functional beverage based on exotic fruits (mango juice, papaya juice and açaí) mixed with orange juice and oat with the addition of *Stevia rebaudiana*, no substantial effect was detected during salivary and gastric phases on any of the main polyphenols, total antioxidant capacity, ascorbic acid, and steviol glycosides, in contrast with carotenoids and anthocyanins that diminished significantly during the gastric phase. All analyzed compounds were significantly affected during the pancreatic-bile digestion being more marked for carotenoids and total anthocyanins, but polyphenols, anthocyanins, total antioxidant capacity and steviol glycosides bioaccessibility increased as did Stevia concentration, whereas ascorbic acid's was negatively affected by Stevia addition (Carbonell-Capella et al. 2015).

Red grape pomace polyphenols (red grape pomace aqueous extract) have also been studied by in vitro and in vivo analyses in IPEC cells (intestinal porcine epithelial cells) and in the duodenum and colon of piglets fed, respectively, to check the correlation between in vitro and in vivo absorption of polyphenols. O-quinones and dimers were found in the cellular and extracellular medium as grape pomace polyphenols oxidation products. Major polyphenols were procyanidin trimer and a procyanidin dimer. As to in vivo studies, in duodenum and colon grape pomace piglet's diet (5%) showed an augment in the antioxidant status and decreased lipid peroxidation (TBARS), and increased SOD activity in duodenum and CAT and GPx activity in the colon (Chedea et al. 2018). Oxidative stress can display negative effects on the intestinal tract through the exposure to luminal ROS from oxidized food debris,

saliva oxidants, toxins, high levels of iron ions, bacteria and bile acids, being of extreme importance for the colon, where residence time is prolonged. Endogenous cellular defense (including the antioxidant enzymes, such as SOD, GPx and CAT) is often exceeded by ROS production needing the contribution of polyphenols (Chedea et al. 2018). In a study conducted on piglets fed with red grape pomace (5%) polyphenols absorption was reported, showing a structural modification of polyphenols by metabolization, and the absorption in the liver, spleen and kidneys in this form, accompanied by the increase in the antioxidant status (Chedea et al. 2019). There has been evidence of a reduction in the phenolic compounds profile of the Merlot grape pomace extract by in vitro digestion process, whereas simulated colonic fermentation had a positive effect over the extract's antiproliferative potential tested on MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), and HepG2 (hepatocellular carcinoma) cells (Corrêa et al. 2017). Red grape pomace (Tempranillo grapes) has been fund to be stable at the stomach and to reduce anthocyanins and flavonols at the small intestine, preserving high antioxidant capacity (evaluated by FRAP, ABTS, and ORAC assays) after storage and in vitro digestion (Wang et al. 2017a). Bioaccessibility studies on different varieties of red grapes showed a reduction in antioxidant capacity after the digestion process because of an important loss of polymeric compounds, being lower for the Tannat variety, which could be related to its higher content of phenolic compounds after digestion. The effect of digestion was critical for anthocyanins, although it was also pronounced in flavanols, regardless of the grape variety of origin. As to flavonols, the changes were generally less pronounced, with high amounts of quercetin-3-O-glucuronide and quercetin-3-O-glucoside after digestion and phenolic acids underwent important changes in their composition. Tannat extract showed a marked decrease in caftaric acid. On the contrary, vanillic acid and syringic acid showed significant increases (Nieto Fuentes 2015). The bioaccessibility of grape pomace and its aqueous extract from white Pinot noir wine was studied by determining polyphenols content, antioxidant capacity and polyphenolic profile after a human digestion simulation, finding higher phenolic content and antioxidant capacities in the extract. The main bioaccessible phenolic compounds were gallic, vanillic and siringic acids, showing the extract higher bioactive value (Beres et al. 2019).

Red grape bioaccessibility has shown increased bioaccessibility of total polyphenols that are already bioaccessible in wine in mouth and stomach digestion while intestinal digestion reduced polyphenol bioaccessibility, being anthocyanins the less affected, along with antioxidant capacity reduction (Lingua et al. 2018). The bioaccessibility of peel, pulp, and seeds of *Vitis labrusca L.* grapes has been studied through in vitro simulation of gastrointestinal digestion showing variations in the bioaccessibility of bioactive compounds between the different digestion phases, with maintained concentrations of hydroxybenzoic and hydroxycinnamic acids, anthocyanins, flavanols, and flavonols, as well as the antioxidant potential, suggesting high bioaccessibility of most phenolic compounds (Gomes et al. 2019). The bioaccessibility of red grape skin and seeds extracts has been studied by Pešić et al. (2019) finding almost two times lower recovery after digestion of total non-flavan-3-ol phenolics extracted from red grape seed compared to red grape skin

extract, probably because of the release of hydroxycinnamic acids (caffeic and p-coumaric) from the grape skin extract. It was detected a considerable loss of flavan-3-ols total recovery and a significant reduction of proanthocyanidin content during digestion of red grape seed extract. The deacylation of anthocyanidin mono- and diglucosides was also detected generating p-coumaric and caffeic acids as well as malvidin-3,5-di-O-glucoside during digestion of red grape skin extract (Pešić et al. 2019).

Anthocyanins bioavailability is subjected to intestinal epithelium physical and physiological barrier (composed of a mucus barrier and cell layer), mainly entering the enterocytes by passive diffusion, or via active transport in a lesser extent, when arriving at their surface in the cell layer for further absorption, enterocyte biotransformation and transportation to the liver for metabolization, or degradation by microbiota when not absorbed in the small intestine (Gadioli Tarone et al. 2020). Regarding polyphenols bioavailability, including anthocyanins, a study of cranberry (*Vaccinium macrocarpon*) juice consumption in healthy older adults showed antioxidant activity in plasma assessed by ORAC and TAP assays, which correlated with individual metabolites bioavailability (Mckay et al. 2015).

Bioavailability studies through Caco-2 cells of ethanolic extracts from the different varieties (white and red grapes) indicated that Tannat bioavailable fraction showed the highest antioxidant capacity with a TEAC value of 12.34 mmol of trolox/L extract, due to the presence of phenolic acids and flavonols. After intestinal absorption, the bioavailable fraction was mainly characterized by the presence of phenolic acids, with an important content of syringic and caftaric acid, together with smaller notable amounts of quercetin-3-O-glucoside and trans-piceid to a lesser extent. In addition, small amounts of p-cumaric acid and quercetin-3-O-glucuronide were detected in this fraction. Grape seed extracts presented the highest phenolic content and antioxidant activity in the bioavailable fraction after intestinal absorption assays. Thus, wine by-products were found to be important sources of bioavailable phenolic compounds (Nieto Fuentes 2015).

Proanthocyanidins bioavailability relies on the polymerization degree, skin having a higher grade of polymerization than seeds, and can reach tissues such as the connective tissue, lung, kidney and spleen, but most reach the colon in an intact state, which preserve intestinal barrier integrity through anti-inflammation activity and antioxidant capacity, among other mechanisms (Unusan 2020). It has been stated that some polyphenols such as piceido are absorbed through the glucose-dependent transporter SGLT1, generally involved in the transport of glycosylated flavonoids, such as quercetin-3-O-glucoside, being bioavailable. In the case of Tannat grape skin ethanolic extract, anthocyanins malvidin-3-O-glucoside, as well as cyanidin and petunidin derivatives, were bioavailable (Nieto Fuentes 2015). Anthocyanins absorption through intestinal epithelial cells may involve GLUT2 transporter when tested on Caco-2 cells (Faria et al. 2009). Talavéra et al. (2004) found that a high proportion of anthocyanin glycosides was absorbed through the small intestine of rats and the rate of absorption depended on the chemical structure of the anthocyanin and varied from 10.7% (malvidin 3-glucoside) to 22.4% (cyanidin 3-glucoside). The study also showed that anthocyanins are quickly metabolized

and present bile and urine excretion as intact glycosides, methylated forms and glucuronidated derivatives (Talavéra et al. 2004). Moreover, 500 mg of aronia berry extract by 6 adults showed anthocyanins bioavailability, an increment of microbial phenolic catabolites (approximately 10-fold more than anthocyanins) in plasma and urine, rapid metabolization of cyanidin-3-O-galactoside into peonidin-3-O-galactoside, and total bioavailability and metabolism of anthocyanins at 24 h (Xie et al. 2016). Still, more studies of grape by-products (pomace, seeds, skin and stem) polyphenols are necessary, mostly of bioavailability studies on other cell types such as normal epithelial small intestinal cells as well as normal epithelial colon cells.

11.5 Food Applications

As previously stated, winemaking byproducts are a source of dietary fiber and polyphenols, and so food industry could use dietary fiber for its physicochemical properties such as organoleptic (texture, viscosity, among others) and sensory characteristics, water retention capacity and prolongation of freshness as well as extending food products shelf-life by polyphenols (Foschia et al. 2013; Iriondo-Dehond et al. 2018; Tseng and Zhao 2013). The most important factor affecting the safety and shelf-life of fruits and vegetables as well as food products is microbial spoilage (Salehi and Aghajanzadeh 2020). For some food products such as meat, it is known that lipids present high susceptibility to peroxidation during cooking and gastrointestinal digestion, forming lipid oxidation products, which may represent negative health effects (Pešić et al. 2019). It is also known that during gastric digestion, lipid rich-foods cause the generation of ROS, which lead to lipid peroxidation, co-oxidation of vitamins, dietary proteins amino acid oxidation of side chains, the formation of protein-protein cross-linkages, and protein fragmentation, reaching the blood when absorbed with the possibility of consuming plasma antioxidants (Urquiaga et al. 2018). As to food processing, such as cooking or boiling, polyphenols stability should be considered before deciding which food product would be ideal for the incorporation of any functional ingredient (Fig. 11.3). Plant cells are broken during heat treatments and chewing with the subsequent release of polyphenols which may interact with cell wall material (Giusti et al. 2019).

Through soaking water processing, anthocyanins, flavonoids and tannins have shown to leach, as well as gallic acid. In the case of boiling water, some polyphenols may be released from food matrix compounds such as anthocyanins and can also be lost like delphinidin 3-glucoside possibly because of thermal degradation of anthocyanins. In the same way, the cooking process caused a reduction of free and bound phenolic compounds content by thermal degradation, but the food matrix can protect thermally labile polyphenols such as anthocyanins (Giusti et al. 2019). Moreover, the effects of baking conditions and dough formulations on polyphenols stability of cookies made from anthocyanin-rich corn flour showed an increase on total flavonoids and anthocyanins content by the addition of citric acid in the cookies prepared from blue popping corn and blue-standard corn, and also an increase by

Fig. 11.3 Food applications of red grape by-products for improving shelf-life, physicochemical properties, food processing effects, and bioaccessibility of bioactive compounds

baking at 150 °C for 7 min compared to 200 °C for 10 min (control cookies) (Žilić et al. 2016). Also, baking conditions reduced free water-soluble polyphenols (total flavonoids and anthocyanins) content in control corn cookies. However, antioxidant capacity was reduced because of Maillard reaction inhibition at low pH with 0.5 and 1 g/100 g citric acid of anthocyanins-rich blue popping corn and blue standard corn cookies (Žilić et al. 2016).

Anthocyanins degradation by food processing can be improved by encapsulation prolonging half-life. Barberry (*Berberis vulgaris*) extract as a rich source of anthocyanins, when encapsulated with three different wall materials which include a combination of Arabic gum and maltodextrin, a combination of maltodextrin and gelatin, and maltodextrin by the spray drying process, all increased anthocyanins half-life storage compared to non-encapsulated ones. The combination of Arabic gum and maltodextrin lowered degradation of anthocyanins in all the tested temperatures and was found as the most effective wall material in stabilizing the pigments. The encapsulated pigments were used as natural colorants for jelly powder instead of synthetic color, finding that the addition of 7% encapsulated color presented higher scores than the commercial jelly containing synthetic color for the sensory attributes evaluated as well as better rheological jelly properties (syneresis and solubility) (Akhavan Mahdavi et al. 2016).

The addition of Chardonnay white grape skin to tomato puree (3%) and to a flatbread (10%), resulted in phenolics enriched foods except for the higher mass proanthocyanidin oligomers (firstly because of binding to food matrix and secondly to heat degradation) which was detected a higher mammalian α-amylase and α-glucosidase (from rat intestine) inhibition for the enriched foods than for unfortified foods. The expected increase in the inhibition was lower in the case of flatbread probably because of the binding of the higher mass proanthocyanidin to the food matrix. Although phenolics interactions with the food matrix could negatively affect bioavailability, the digestion process can enhance bioavailability by releasing them from the food matrix. The amount of white grape skin added to both food products was stated by previous sensory analysis with consumers. Hence, both enriched foods may potentially alleviate the damage caused by hyperglycemia (Lavelli et al. 2016).

Red wine ("Xueyuanpai" red wine Cabernet Sauvignon) polyphenol bioaccessibility has been studied by in vitro gastrointestinal digestion showing a good release of polyphenols at mouth and stomach (release rates of 88.59% to 95.86% at the stomach) steps, and after stomach digestion, a release rate of 40–50% was reported at the "serum-available" fraction (foodstuffs at the small intestine and are absorbed), while others were released in a rate of 20% in the "colon-available" fraction, serving as substrates for gut microbiota as well as influencing microbiota ecosystem or continuing the absorption into the serum (Sun et al. 2020). In the same study, decrease in the inhibitory effects of α-amylase and α-glucosidase along digestion steps was reported, and a moderate wine intake and drinking after the meal were suggested because of showing a higher serum and lower colon-available total polyphenol value than drinking before a meal in all three wine drinking amounts (Sun et al. 2020).

In a study conducted on red wine grape pomace (*Vitis vinifera* L. cv. Pinot Noir), it was demonstrated that wine grape pomace could be added to yogurt and as a salad dressing as a source of dietary fiber and polyphenols. Its addition caused the extending shelf-life of food products and the possibility to be used as a functional food ingredient for promoting human health. Its addition decreased yogurt viscosity and syneresis was not observed in 4 week storage time except for 3% addition (Tseng and Zhao 2013). In the case of fortified yogurt, there were no significant differences between control, 1% WP and 2% WP (w/w yogurt) samples in appearance liking and overall liking, but 2% WP yogurt received a lower score on flavor and texture liking. As to fortified Italian dressing there was no difference ($P > 0.05$) on all measured sensory attributes for control, 0.5% WP and 1% WP and for fortified Thousand Island dressing there was no significant difference ($P > 0.05$) on appearance, overall and flavor liking for control, 1% WP and 2% WP (Tseng and Zhao 2013). Red grape skins and grape seeds incorporation (0, 5, 10, and 15% to the weight of flour) in cookies resulted in changed rheological parameters (increased water absorption and reduced dough stability for grape skin and the opposite for grape seeds, reduced volume, thickness, hardness, and fracturability) along with good overall acceptability for 5%-enriched cookies (Kuchtová et al. 2018). To extend fish shelf-life during storage by delaying lipid oxidation because of high unsaturated lipid content that is

very susceptible, red grape antioxidant dietary fiber was added to minced horse mackerel (*Trachurus trachurus*) fish muscle finding considerably delayed lipid oxidation during the first 3 months of frozen storage (Sánchez-Alonso et al. 2006). Grape seed flour has been incorporated into frankfurters resulting in a decline in the oxidation level of the products (Zhu et al. 2015). Red grape skin has been used as a seasoning of marinated chicken breasts as a salt replacer obtaining the same shelf-life of 0.5% replace of salt and 2% of seasoning than the one with 2% of salt but presenting lower sensory scores in color, texture and overall linking (3.1–3.3) and related to consumers' willingness to accept new products (Ortega-Heras et al. 2020). Five formulations of whole-wheat muffins with white and red grape pomace were studied (100% whole-wheat flour control muffin, muffins+10% white pomace, muffin+20% white pomace, muffin+10% red grape pomace, and muffin+20% red grape pomace) with "high-fiber content", leading to changes in sensory attributes (decrease in cohesiveness, springiness, resilience and color, and increase in hardness and chewiness) but with high acceptability levels in muffin+10% grape pomace, representing a healthier alternative for muffins formulations development (Ortega-Heras et al. 2019). Grape pomace can also be incorporated into bakery products such as cakes that are worldwide consumed but organoleptic properties, texture, and color are affected, as a consequence of insoluble and soluble fiber that can enhance cake technological attributes as water binders, gelling agents, fat replacers and texture improvers, which may improve cake quality (preventing the augment of air bubbles which are incorporated toward the surface during the baking process and increasing viscosity of the batter). Grape pomace powder has been found as a suitable replacer of wheat flour in muffin formulations (Salehi and Aghajanzadeh 2020). Particularly, Riesling and Tannat skin flour as wheat flour replacers in muffins (5, 7.5, and 10%) showed decreased lightness of the flour, cohesiveness value, as well as increased the lightness values (L*) of the muffin crumbs and crusts and hardness of muffins with an increased percentage of the skin flour replacement, with no changes in color, taste, flavor, texture, and overall acceptability (from 5.2 to 5.7 on a 7-point hedonic scale) of the muffins determined by sensory analysis, resulting in suitable alternative to increase the dietary fiber content of muffins (Bender et al. 2017). Red Traminer, Alibernet, and Cabernet grape pomaces (3%) were found suitable for gluten-free products (muffins and knäckebrots), being Cabernet pomace muffins the ones with the highest sensory quality (Matejová et al. 2019).

Besides techno-functional properties, wine grape by-products could have a health-promoting effect by their addition to different food products, in which case structure and composition of the food product's matrix effect on the bioaccessibility of grape by-products bioactive compounds should be considered. Food product's matrix can impair/enhance the release and stability of grape bioactive compounds during digestion compromising their biological effect (Iriondo-Dehond et al. 2018; Pineda-Vadillo et al. 2016). Dairy and egg products are excellent foods to be fortified because of worldwide acceptance by all age groups, nutritional properties, which could be represented by numerous forms and structures and can be eaten daily (Pineda-Vadillo et al. 2016). Yogurt formulation enriched with grape pomace aqueous extract from white wine Pinot noir was tested for sensory panelists

resulting in an overall liking score of 6.2 out of 9.0 and 51% of panelists would buy the product. The incorporation of the extract in yogurt was found as a potential antioxidant dietary fiber ingredient (Beres et al. 2019). Antioxidant dietary fiber from red grape pomace has been reported as promising sources of functional ingredients for enriched meat-based functional foods by ameliorating oxidative changes in meat products and providing health benefits. Nevertheless, physical characteristics change because of grape pomace addition to meat products, such as color in raw and cooked chicken hamburgers but sensory values were improved (0.5%, 1.0%, 1.5% and 2.0% of grape pomace addition) (Das et al. 2020). Red grape pomace (Vitis vinifera L cv. Corvina) has also been used to fortified durum wheat pasta (spaghetti) replacing semolina with 0, 5, and 10 g/100 g of grape pomace, enhancing total polyphenol content and antioxidant capacity (ABTS and FRAP values) accompanied by the reduction of cooking time and swelling index, enhanced firmness and adhesiveness of the pasta, and good overall acceptability (Tolve et al. 2020). However, there are few bioaccessibility studies on enriched food products with grape by-products such as dairy, bakery and egg products (Pešić et al. 2019). Pešić et al. (2019) studied the bioaccessibility of bioactive compounds in an infant puree composed of turkey meat, potato, corn and rice that was enriched with red grape skin and seed extracts. Polyphenols recovery of red grape skin extract's addition to the food matrix was not affected in contrast with their digestion without food matrix, mostly because of food matrix polyphenols contribution to total polyphenols content before digestion. On the other hand, the addition of red grape seed extract to infant puree increased the total recovery of flavan-3-ols and non-flavan-3-ol compared to the digestion of the extract alone. Digestion of red grape extracts with infant puree showed higher total phenolic content, finding better antioxidant capacity in the grape skin extract than in red grape seeds extract due to flavan-3-ols binding capacity with antioxidant components from food matrix and digestive fluids. Moreover, anthocyanins stability was decreased by the food matrix resulting in the disappearance of malvidin-3-O-glucoside and to a lower extent the release of malvidin-3,5-di-O-glucoside in the final digest compared to that in digested grape skin extract (Pešić et al. 2019). Red grape marc enriched durum wheat spaghetti improved the amount of phenolic compounds and antioxidant capacity, and the bioaccessible fraction showed higher amount of polyphenols including anthocyanins and antioxidant capacity but a lower amount of glucose with sensory acceptance (Marinelli et al. 2018).

In other polyphenols bioaccessibility study conducted on dairy and egg products enriched with red grape extracts (custard dessert, milkshake, pancake and omelet), results showed a great impact on anthocyanins and proanthocyanidins release and solubility during digestion (Pineda-Vadillo et al. 2016). The biggest impact was detected in solid food matrices and in oral and gastric digestion phases as well as showing protection of the degradation of anthocyanins by food matrices during the intestinal digestion phase. Moreover, antioxidant activity remained constant during oral and gastric phases, increasing during the intestinal phase. Comparing all food matrices, omelet presented the highest total phenolics and antioxidant activity (FRAP antioxidant activity and ORAC-FL) recoveries at the end of digestion. In

pancake and omelet anthocyanins, proanthocyanidins and total phenolics were mostly recovered in the insoluble fraction during oral and gastric digestion phases, and anthocyanins were actually protected from degradation during intestinal digestion phase (Pineda-Vadillo et al. 2016).

Another source of anthocyanins (black carrot pomace) was added to cake and studied bioactive compounds bioaccessibility, finding no difference between the 100 g/kg and 150 g/kg pomace addition after digestion in the content of polyphenols. During oral and gastric phases the amount of anthocyanins and phenolic acids were reduced significantly, and after intestinal phase anthocyanins were not detected, but total phenolic content and total antioxidant capacity were increased during gastric and intestinal phases (up to 5- and 12-fold respectively) (Kamiloglu et al. 2017).

In addition to bioaccessibility studies of grape pomace by-products in different food matrices, more bioavailability and/or in vivo bioactivity studies are needed to ensure grape byproducts effects on human health as a consequence of the interactions between grape by-products and different food matrices that could have a positive or negative effect on bioavailability with the subsequent effect on in vivo bioactivity. In a three-month intervention study conducted on 27 male volunteers, each with some components of metabolic syndrome, Cabernet sauvignon grape pomace meat burgers were formulated with 7% of containing 3.5% fiber, 1.2 mg GE/g of polyphenols, and 17.2 μmol TE/g of ORAC compared to raw control-burger containing no fiber, 0.396 mg GE/g of polyphenols, and 1.82 μmol TE/g of ORAC (Urquiaga et al. 2018). Cabernet Sauvignon grape pomace burger intake showed an increase in plasma of the essential contributor to plasma antioxidant defense vitamin C, compared to control-burger intake decreased concentration (up to baseline levels) (Urquiaga et al. 2018). Moreover, grape pomace burger intake showed a significant reduction of glycemia and HOMA index values (a measurement of insulin resistance), as well as significantly decreased advanced oxidation protein products and oxidized low-density lipoprotein levels, finding potential as a functional ingredient for the prevention and/or treatment of diabetes mellitus and cardiovascular disease (Urquiaga et al. 2018).

11.6 Conclusions

Vast evidence of the polyphenolic composition of red wines and by-products is available, especially for anthocyanins, but there is scarce knowledge of the by-products bioactivities after digestion, including antioxidant, anti-inflammatory, anti-diabetic, and anti-obesity, among others. Furthermore, the loss or gain of red grape by-products bioactivity during gastrointestinal digestion, colonic fermentation process, and intestinal barrier passage should be further studied to be considered for the development of new functional foods. Also, the by-products addition to food products should be studied regarding sensory analysis to achieve consumers' acceptance, along with bioaccessibility and bioavailability of the new red wine

byproduct-added functional foods, considering the variable effects of food matrix on these accounts, determining bioactive properties other than antioxidant to ensure a global approach on the risk reduction of chronic diseases.

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