Methods and Protocols in Food Science

Springer Protocols

Adriano Gomes da Cruz · Marcia Cristina Silva
Tatiana Colombo Pimentel
Erick Almeida Esmerino · Silvani Verruck
Editors

Probiotic Foods and Beverages

Technologies and Protocols

Methods and Protocols in Food Science

Series Editor
Anderson S. Sant'Ana
University of Campinas
Campinas, Brazil

For further volumes:
http://www.springer.com/series/16556

Methods and Protocols in Food Science series is devoted to the publication of research protocols and methodologies in all fields of food science.

Volumes and chapters will be organized by field and presented in such way that the readers will be able to reproduce the experiments in a step-by-step style. Each protocol will be characterized by a brief introductory section, followed by a short aims section, in which the precise purpose of the protocol will be clarified.

Probiotic Foods and Beverages

Technologies and Protocols

Edited by

Adriano Gomes da Cruz

Department of Food, Federal Institute of Science, Rio de Janeiro, Brazil

Marcia Cristina Silva

Department of Food, Federal Institute of Science, Rio de Janeiro, Brazil

Tatiana Colombo Pimentel

Federal University of Paraná, Paranavaí, Brazil

Erick Almeida Esmerino

Department of Food Technology, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

Silvani Verruck

Department of Food Science and Technology, Federal University of Santa Catarina, Florianópolis, Brazil

Humana Press

Editors
Adriano Gomes da Cruz
Department of Food
Federal Institute of Science
Rio de Janeiro, Brazil

Marcia Cristina Silva
Department of Food
Federal Institute of Science
Rio de Janeiro, Brazil

Tatiana Colombo Pimentel
Federal University of Paraná
Paranavaí, Brazil

Erick Almeida Esmerino
Department of Food Technology
Federal University of Rio de Janeiro
Rio de Janeiro, Brazil

Silvani Verruck
Department of Food Science
and Technology
Federal University of Santa Catarina
Florianópolis, Brazil

ISSN 2662-950X ISSN 2662-9518 (electronic)
Methods and Protocols in Food Science
ISBN 978-1-0716-3186-7 ISBN 978-1-0716-3187-4 (eBook)
https://doi.org/10.1007/978-1-0716-3187-4

© The Editor(s) (if applicable) and The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2023, Corrected Publication 2023
This work is subject to copyright. All rights are solely and exclusively licensed by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed.
The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.
The publisher, the authors, and the editors are safe to assume that the advice and information in this book are believed to be true and accurate at the date of publication. Neither the publisher nor the authors or the editors give a warranty, expressed or implied, with respect to the material contained herein or for any errors or omissions that may have been made. The publisher remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This Humana imprint is published by the registered company Springer Science+Business Media, LLC, part of Springer Nature.
The registered company address is: 1 New York Plaza, New York, NY 10004, U.S.A.

Preface to the Series

Methods and Protocols in Food Science series is devoted to the publication of research protocols and methodologies in all fields of food science. The series is unique as it includes protocols developed, validated and used by food and related scientists, as well as theoretical basis are provided for each protocol. Aspects related to improvements in the protocols, adaptations and further developments in the protocols may also be approached.

Methods and Protocols in Food Science series aims to bring the most recent developments in research protocols in the field as well as very well established methods. As such the series targets undergraduate, graduate and researchers in the field of food science and correlated areas. The protocols documented in the series will be highly useful for scientific inquiries in the field of food sciences, presented in such way that the readers will be able to reproduce the experiments in a step-by-step style.

Each protocol will be characterized by a brief introductory section, followed by a short aims section, in which the precise purpose of the protocol is clarified. Then, an in-depth list of materials and reagents required for employing the protocol is presented, followed by a comprehensive and step-by-step procedures on how to perform that experiment. The next section brings the do's and don'ts when carrying out the protocol, followed by the main pitfalls faced and how to troubleshoot them. Finally, template results will be presented and their meaning/conclusions addressed.

The Methods and Protocols in Food Science series will fill an important gap, addressing a common complain of food scientists, regarding the difficulties in repeating experiments detailed in scientific papers. With this, the series has a potential to become a reference material in food science laboratories of research centers and universities throughout the world.

Campinas, Brazil ***Anderson S. Sant'Ana***

Preface

The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have defined probiotics as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. The global probiotics market was valued at USD 58.17 billion in 2021 and is expected to expand at a compound annual growth rate (CAGR) of 7.5% from 2021 to 2030. The health promotion provided by these microorganisms has been the main driving force of this market niche. Also, an emerging functional food discipline in this field is using postbiotics and paraprobiotics in food and beverages. Paraprobiotics and postbiotics can express health benefits in addition to the inherent viability of probiotics, proving that not all mechanisms, nor clinical effects, are directly related to viable bacteria and broadening the current concept of what probiotics are. Furthermore, paraprobiotics and postbiotics have valuable potential for developing biotechnological products with functional ingredients and are more stable, allowing for easier use on an industrial scale.

Protocols in Technology of Probiotic Foods and Beverages is a book that addresses the latest relevant state-of-the-art protocols to manufacture functional probiotic foods and beverages. In addition, this book combines, as comprehensibly as possible, well-established protocols and procedures used by many laboratories in academia and industry.

Regarding dairy products, Chap. 1 provides information about the material, main processing procedure, and packaging steps for processing fermented milks. At the same time, Chap. 2 discusses probiotic strains used to manufacture different cheese types and the survival of those probiotics, regarding actions taken to increase their viability. The limitations from research to industrial limitations and the main factors to consider for appropriate probiotic strain selection for industrial application are pointed out. Chapter 3 is a practical guidance for probiotic ice cream manufacture, presenting the steps and amount of probiotic addition into ice cream production. Finally, Chap. 4 is a practical guidance for probiotic butter manufacture, discussing ways of adding probiotics.

Regarding non-dairy products, Chap. 5 deals with plant-based beverages, demonstrating the process of obtaining soy, oat, and rice extracts and the fermentation process to obtain probiotic beverages. At the same time, Chap. 6 describes the process of obtaining probiotic plant-based cheeses, such as pea cheese, tofu, soy-based cream cheese, and chickpea petit Suisse cheese. Chapter 7 describes a method incorporating probiotic bacteria encapsulated in an alginate matrix using an emulsification process as a pretreatment into fruit juices. Furthermore, techniques for morphological analysis by scanning electron microscopy, as well as the characterization of the juice and the evaluation of cell viability against simulated gastric conditions, are provided. Chapter 8 describes the process of obtaining probiotic-fermented vegetables, such as pickles, sauerkraut, and natto. Chapter 9 describes two preparation methods of Kombucha using a symbiotic culture of bacteria and yeast or a synthetic microbial community as a starter. Moreover, the determination of bioactive compounds, including organic acids, sugars, and catechins, has been introduced. Chapter 10 provides a guideline on preparing a probiotic beer that can be used for

researching new probiotic microorganisms and highlights essential points to be considered when developing probiotic beers. Chapter 11 describes a protocol for probiotic Friolano-type sausage. Furthermore, the possible sources of defects in producing probiotic salami and the best alternatives to overcome them are presented. Chapter 12 proposes the design of two independent protocols for the delivery of probiotics through bakery products: (I) a probiotic bread by adding microorganisms directly to the dough and (II) an edible probiotic film based on sodium caseinate and chia mucilage for application in bread surface. Furthermore, for both protocols, the function of each reagent/ingredient and the chemical reactions involved are described in detail, indicating the possible issues, sources, and the best alternatives to overcome them. Finally, Chap. 13 has up-to-date and detailed information on the production of different probiotics and synbiotic chocolate.

Regarding proposals for increasing probiotic survival in food products, Chap. 14 describes in detail the main methods of encapsulation of probiotics, including emulsion, extrusion, and spray-drying techniques.

However, in recent years, researchers have observed that viability may not be necessary for some health effects, and products with inactivated microorganisms have been developed. In this way, Chaps. 15 and 16 provide detailed protocols for obtaining potential paraprobiotics and postbiotics for use in food and beverages.

Finally, following new health effects associated with probiotic cultures, Chap. 17 describes protocols for elaborating on a food product with psychobiotic potential in detail. In addition, the most used behavioral tests for preclinical trials that can be applied to confirm the psychobiotic effect are also discussed.

Rio de Janeiro, Brazil — *Adriano Gomes da Cruz*
Rio de Janeiro, Brazil — *Marcia Cristina Silva*
Paranavaí, Brazil — *Tatiana Colombo Pimentel*
Rio de Janeiro, Brazil — *Erick Almeida Esmerino*
Florianópolis, Brazil — *Silvani Verruck*

Contents

Preface to the Series . . . . *v*
Preface . . . . *vii*
Contributors . . . . *xi*

1 Probiotic Fermented Milk 1
Shibo Ma, J. K. Vidanarachchi, and Chaminda Senaka Ranadheera

2 Probiotic Cheeses 35
Celso Fasura Balthazar, Julien Chamberland, and Marie-Claude Gentès

3 Probiotic Ice Creams 53
Vanessa Cortina Zanetti, Celso Fasura Balthazar, Callebe Camelo-Silva, and Silvani Verruck

4 Probiotic Butter 67
Patrícia Blumer Zacarchenco, Leila Maria Spadoti, Adriana Torres Silva e Alves, Vanessa Cortina Zanetti, and Silvani Verruck

5 Probiotic Plant-Based Beverages 81
Tahis R. Baú, Danielle C. B. H. Ferreira, Cintia L. Handa, Fernando S. de Lima, and Tatiana Colombo Pimentel

6 Probiotic Plant-Based Cheese 93
Tahis R. Baú, Danielle C. B. H. Ferreira, Cintia L. Handa, Fernando S. de Lima, and Tatiana Colombo Pimentel

7 Probiotic Fruit Juices 105
Oscar O. Romero-Chapol, Abigail Varela-Pérez, Ana G. Castillo-Olmos, Hugo S. García, and Cynthia Cano-Sarmiento

8 Probiotic Fermented Vegetables 119
Hadi Pourjafar, Tatiana Colombo Pimentel, and Tahis R. Baú

9 Kombucha Production and Its Bioactive Compounds Analysis 133
Chun Zou, Yong-Quan Xu, Yi-Bin Huang, and Jun-Feng Yin

10 Probiotic Beer 139
Fernanda Meybom, Bárbara Mortl, and Alan Ambrosi

11 Probiotic Fermented Sausage 151
Claudio M. E. Malaghini, Ana Paula Zapelini de Melo, and Silvani Verruck

12 Probiotic in Bakery 165
Ana Paula Zapelini de Melo, Thais de Oliveira, Pedro Luiz Manique Barreto, and Silvani Verruck

13 Probiotic and Synbiotic Chocolate 179
Milena Dutra Pierezan, Callebe Camelo-Silva, Alan Ambrosi, Marco Di Luccio, and Silvani Verruck

14 Microencapsulation of Probiotics 199
Callebe Camelo-Silva, Lais Leite Figueredo, Vanessa Cortina Zanetti, Alan Ambrosi, Marco Di Luccio, and Silvani Verruck

15 Paraprobiotics Preparation for Use in Food and Beverages 213
Cássia Pereira Barros, Roberto Pessanha da Silva Pires, and Jonas de Toledo Guimarães

16 Postbiotics Preparation for Use in Food and Beverages 223
Jonas de Toledo Guimarães, Cássia Barros, Houshmand Sharafi, Mehran Moradi, Erick A. Esmerino, and Adriano Gomes da Cruz

17 Psychobiotic Carried by Food and Beverage. 243
Cássia Pereira Barros, Erick A. Esmerino, Roberto Laureano Melo, and Adriano Gomes da Cruz

Correction to: Paraprobiotics Preparation for Use in Food and Beverages C1
Cássia Pereira Barros, Roberto Pessanha da Silva Pires, and Jonas de Toledo Guimarães

Index *263*

Contributors

ALAN AMBROSI • *Graduate Program in Food Engineering, Federal University of Santa Catarina, Florianópolis, SC, Brazil; Graduate Program in Food Science, Federal University of Santa Catarina, Florianópolis, SC, Brazil*

CELSO FASURA BALTHAZAR • *Department of Food Science and Nutrition, Faculty of Food Engineering, University of Campinas, Campinas, SP, Brazil*

PEDRO LUIZ MANIQUE BARRETO • *Graduate Program in Food Science, Federal University of Santa Catarina, Florianópolis, SC, Brazil*

CÁSSIA PEREIRA BARROS • *Department of Food Technology, Faculty of Veterinary Medicine, Federal Fluminense University (UFF), Niterói, Rio de Janeiro, Brazil*

TAHIS R. BAÚ • *Federal Institute of Santa Catarina, Campus São Miguel do Oeste, São Miguel do Oeste, Santa Catarina, Brazil*

CALLEBE CAMELO-SILVA • *Graduate Program in Food Science, Federal University of Santa Catarina, Florianópolis, SC, Brazil; Graduate Program in Food Engineering, Federal University of Santa Catarina, Florianópolis, SC, Brazil*

CYNTHIA CANO-SARMIENTO • *CONACYT—Tecnológico Nacional de México/Instituto Tecnológico de Veracruz, Unidad de Investigación y Desarrollo en Alimentos, Veracruz, Mexico*

ANA G. CASTILLO-OLMOS • *CONACYT—Tecnológico Nacional de México/Instituto Tecnológico de Veracruz, Unidad de Investigación y Desarrollo en Alimentos, Veracruz, Mexico*

JULIEN CHAMBERLAND • *STELA Dairy Research Center, Institute of Nutrition and Functional Foods (INAF), Department of Food Science and Nutrition, Faculty of Agricultural Science and Food, University Laval, Québec City, QC, Canada*

ADRIANO GOMES DA CRUZ • *Department of Food Science and Technology, Agrarian Science Center, Federal University of Santa Catarina (UFSC), Florianópolis, SC, Brazil; Department of Food, Federal Institute of Education, Science and Technology of Rio de Janeiro (IFRJ), Rio de Janeiro, Brazil*

FERNANDO S. DE LIMA • *Federal Institute of Paraná, Campus Paranavaí, Paraná, Brazil*

ANA PAULA ZAPELINI DE MELO • *Graduate Program in Food Science, Federal University of Santa Catarina, Florianópolis, SC, Brazil*

THAIS DE OLIVEIRA • *Graduate Program in Food Science, Federal University of Santa Catarina, Florianópolis, SC, Brazil*

JONAS DE TOLEDO GUIMARÃES • *Department of Food Technology, Faculty of Veterinary, Federal Fluminense University, Niterói, RJ, Brazil*

MARCO DI LUCCIO • *Graduate Program in Food Engineering, Federal University of Santa Catarina, Florianópolis, SC, Brazil; Graduate Program in Food Science, Federal University of Santa Catarina, Florianópolis, SC, Brazil*

ERICK A. ESMERINO • *Department of Food Technology, Faculty of Veterinary Medicine, Federal Fluminense University (UFF), Niterói, Rio de Janeiro, Brazil*

DANIELLE C. B. H. FERREIRA • *Federal Institute of Paraná, Campus Jaguariaíva, Paraná, Brazil*

LAIS LEITE FIGUEREDO • *Graduate Program in Food Engineering, Federal University of Santa Catarina, Florianópolis, SC, Brazil*

HUGO S. GARCÍA • *CONACYT—Tecnológico Nacional de México/Instituto Tecnológico de Veracruz, Unidad de Investigación y Desarrollo en Alimentos, Veracruz, Mexico*
MARIE-CLAUDE GENTÈS • *Saint-Hyacinthe Development and Research Centre, Agriculture and Agri-Food Canada, Saint-Hyacinthe, QC, Canada*
CINTIA L. HANDA • *Federal Institute of Paraná, Campus Pitanga, Paraná, Brazil*
YI-BIN HUANG • *Tea Research Institute Chinese Academy of Agricultural Sciences, Hangzhou, China*
CLAUDIO M. E. MALAGHINI • *Graduate Program in Food Science, Federal University of Santa Catarina, Florianópolis, SC, Brazil*
SHIBO MA • *School of Agriculture and Food, Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Parkville, VIC, Australia*
ROBERTO LAUREANO MELO • *Behavioral Physiopharmacology Laboratory, Barra Mansa Center University, Rio de Janeiro, Brazil; Department of Food Technology, Behavioral Physiopharmacology Laboratory, Barra Mansa Center University, Rio de Janeiro, Brazil; Institute of Technology, Federal Rural University of Rio de Janeiro, Rio de Janeiro, Brazil*
FERNANDA MEYBOM • *Regional Council of Engineering and Agronomy of Santa Catarina (CREA), Florianópolis, SC, Brazil; Graduate Program in Food Engineering, Federal University of Santa Catarina, Florianópolis, SC, Brazil*
MEHRAN MORADI • *Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran*
BÁRBARA MORTL • *Kairós Brewery, Florianópolis, SC, Brazil*
ROBERTO PESSANHA DA SILVA PIRES • *Department of Food, Federal Institute of Education, Science and Technology of Rio de Janeiro (IFRJ), Rio de Janeiro, Rio de Janeiro, Brazil; Department of Food Science and Nutrition, Faculty of Food Engineering, University of Campinas (UNICAMP), Campinas, São Paulo, Brazil*
MILENA DUTRA PIEREZAN • *Graduate Program in Food Science, Federal University of Santa Catarina, Florianópolis, SC, Brazil*
TATIANA COLOMBO PIMENTEL • *Federal Institute of Paraná, Campus Paranavaí, Paraná, Brazil*
HADI POURJAFAR • *Alborz University of Medical Sciences, Dietary Supplements, and Probiotic Research Center, Karaj, Iran*
CHAMINDA SENAKA RANADHEERA • *School of Agriculture and Food, Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Parkville, VIC, Australia*
OSCAR O. ROMERO-CHAPOL • *CONACYT—Tecnológico Nacional de México/Instituto Tecnológico de Veracruz, Unidad de Investigación y Desarrollo en Alimentos, Veracruz, Mexico*
HOUSHMAND SHARAFI • *Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran*
ADRIANA TORRES SILVA E ALVES • *Institute of Food Technology ITAL, São Paulo, SP, Brazil*
MARCIA CRISTINA SILVA • *Department of Food, Federal Institute of Education, Science and Technology of Rio de Janeiro (IFRJ), Rio de Janeiro, Brazil*
LEILA MARIA SPADOTI • *Institute of Food Technology ITAL, São Paulo, SP, Brazil*
ABIGAIL VARELA-PÉREZ • *CONACYT—Tecnológico Nacional de México/Instituto Tecnológico de Veracruz, Unidad de Investigación y Desarrollo en Alimentos, Veracruz, Mexico*

SILVANI VERRUCK • *Graduate Program in Food Science, Federal University of Santa Catarina, Florianópolis, SC, Brazil; Department of Food Science and Technology, Agrarian Science Center, Federal University of Santa Catarina (UFSC), Florianópolis, SC, Brazil*

J. K. VIDANARACHCHI • *Department of Animal Science, Faculty of Agriculture, University of Peradeniya, Peradeniya, Sri Lanka*

YONG-QUAN XU • *Tea Research Institute Chinese Academy of Agricultural Sciences, Hangzhou, China*

JUN-FENG YIN • *Tea Research Institute Chinese Academy of Agricultural Sciences, Hangzhou, China*

PATRÍCIA BLUMER ZACARCHENCO • *Institute of Food Technology ITAL, São Paulo, SP, Brazil*

VANESSA CORTINA ZANETTI • *Graduate Program in Food Science, Federal University of Santa Catarina, Florianópolis, SC, Brazil; Graduate Program in Food Science and Technology, Federal University of Santa Catarina, Florianópolis, SC, Brazil*

CHUN ZOU • *Tea Research Institute Chinese Academy of Agricultural Sciences, Hangzhou, China*

Chapter 1

Probiotic Fermented Milk

Shibo Ma, J. K. Vidanarachchi, and Chaminda Senaka Ranadheera

Abstract

Probiotic fermented milk is a product made by appropriate microbial growth using milk as the substrate which contains mainly live microorganisms. Fermented milk has been consumed for thousands of years worldwide, and the incorporation of probiotics has pushed it in a novel direction. The substrate selection includes cows, buffalo, goats, sheep, yak, horses, camel, and others' milk. The various substrate has their uniqueness, and typical traditional products, including kefir, koumiss, etc., are made from them. Further, the range of probiotics is vast, and commonly used genera contain *Lactobacillus* and *Bifidobacterium*. The primarily incorporated method is to inoculate it into the starter culture to co-ferment substrate with traditional fermentation culture. Other methods include fermenting substrate directly or adding it back into the product. The typical products include ambient-temperature fermented milk or probiotic fermented milk beverage. The basic processing method of probiotic fermented milk is similar to traditional fermented milk, where the incorporation of probiotics into the fermented milk product is unique due to the special incubation requirement of each probiotic. Commonly seen additives include sweetener, thickener (thickening technology), and prebiotics which were introduced in this chapter, which could give a comprehensive vision of the current fermented milk production and the indication of applying these additives to the fermented milk considering the existence of probiotics. Some novel and popular fermented milk products and their manufacturing methods were briefly introduced in this chapter, such as ambient-temperature fermented milk, roasted flavor fermented milk, and probiotic fermented milk beverage. General products' quality issues and legal compliance were also mentioned. Still, the most critical way to determine the manufacturing procedure and parameter is by running a pilot test based on the designation of the product, which could give a clear indication of the material, method, and post-manufacturing issues.

Key words Probiotic fermented milk, Manufacture process, Probiotics, Special milk, Sweetener, Prebiotics, Thickening technology

1 Introduction

Probiotic fermented milk is a product derived from traditional fermented milk. Fermented milk is a milk product made via appropriate microbial growth and/or enzymatic conversions of milk [1]. Here, the probiotic fermented milk should go further, where it requires the existence of probiotics in the fermented milk. It was recognized that probiotic fermented milk should contain live

Adriano Gomes da Cruz et al. (eds.), *Probiotic Foods and Beverages: Technologies and Protocols*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-3187-4_1,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2023

microorganisms [2]. However, the recent research regarding parabiotics and postbiotics broadened the scope of the products [3], where the importance of the viability of probiotics had been assimilated. In this chapter, *probiotic fermented milk*, referred in a broad sense, is a cluster of fermented milk products containing probiotic strains, live or not. More detailed introduction about parabiotics and postbiotics will be given in Chaps. 15 and 16. Fermented milk has been consumed for thousands of years. It was originated from various places, such as Mongolia, Egypt, Caucasian areas, etc., where multiple products were developed to fulfill the local requirements. For a clear written record, Greek and Roman are the first to mention this type of product (yogurt) in their history, around 100 BC [4]. For probiotics, its health effect had been realized and applied for dozens of centuries, combined with fermented milk consumption [5]. However, its mystery hadn't been revealed until modern times for their existence and taxonomy. In 1857, Pasteur discovered lactic acid bacteria (LAB) for their role in the fermentation of yogurt. In 1908, Elie Metchnikov proposed the idea of probiotics' health effect in his book *The prolongation of life: optimistic studies* (where the word "probiotic" haven't been proposed yet) [5, 6]. In 1953, German scientist Werner Kollath proposed the term "probiotic," which has been further used [7]. For currently admitted and used probiotic definition and effect, it was determined and published by FAO/WHO in 2001 and slightly modified in 2014 by Hill et al. [8] who confirmed that the *probiotic* should be "live microorganisms which could confer a health benefit on the host, when being administrated in appropriate amount." This definition differed the probiotic fermented milk from other traditional fermented milk (relatively different, traditionally used microorganisms for fermentation were sometimes regarded as probiotic in some situations), where the probiotic in the products should be capable of conferring benefit to humans after consumption. Firstly, the probiotic should tolerate gastric, bile, and intestinal fluid, and could colonize and proliferate in the gastrointestinal tract (GI tract). The safety and viability of probiotics are critical to the selection criteria, where the evaluation procedure has been clarified recently. China has published a new Group Standard names *Probiotic Food* by China National Food Industry Association (CNFIA) to define the requirement of probiotics used in food and the evaluation procedure to evaluate their safety and viability (T/CNFIA 131–2021) (*see* **Notes 1** and **2**) [9]. The standard also requires the precise strain number and source, and the completion of whole genome sequencing and random clinical trial to support its efficacy based on scientific articles. Other scholars also believe the probiotics used in the fermented milk (food) should exist in the GI tract originally, and genetically modified (GM) strain/species should not be used [10]. Meanwhile, there are a lot of strains or species that were tested and claimed to possess probiotic potential. Still, the authorities did not have explicit

consensuses to determine which strain/species or groups can be regarded as probiotics. For example, China and Canada had a list showing the possibility of adding these species into foods as probiotics (Table 1).

Table 1
The list of microbial strains available to be used in foods in China and other countries [89–92]

Genera	Species
Bifidobacterium	*Bifidobacterium adolescentis*[*,^,#] *Bifidobacterium animalis* subsp. *animalis* [*,^,#] *Bifidobacterium animalis* subsp. *lactis*[*,^,#] *Bifidobacterium bifidum*[*,^,#] *Bifidobacterium breve*[*,^,#] *Bifidobacterium longum*[*,^,#] *Bifidobacterium longum* subsp. *Longum*[*,^,#!] *Bifidobacterium longum* subsp. *Infantis* *Bifidobacterium longum* subsp. *Suis*[*,^,#!]
Bacillus (*Assessed* case-by-case in AU)	*Bacillus subtilis*[^!] *Bacillus cereus*[^!]
Companilactobacillus	*Companilactobacillus farciminis* [#!]
Debaryomyce[%]	*Debaryomyces hansenii*[#!]
Enterococcus (*Assessed* case-by-case in AU)	*Enterococcus faecium*[^!] *Enterococcus faecalis*[^!]
Fructilactobacillus	*Fructilactobacillus sanfranciscensis* [#!]
Lacticaseibacillus	*Lacticaseibacillus casei*[#] *Lacticaseibacillus paracasei* [#] *Lacticaseibacillus rhamnosus*[#]
Lactiplantibacillus	*Lactiplantibacillus paraplantarum* [#!] *Lactiplantibacillus plantarum*[#]
Lactobacillus	*Lactobacillus acidophilus*[*,^,#] *Lactobacillus amylolyticus*[*,^,#!] *Lactobacillus crispatus*[*,^,#] *Lactobacillus delbrueckii*[#!] *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lactobacillus bulgaricus*)[#] *Lactobacillus delbrueckii* subsp. *Delbrueckii*[#!] *Lactobacillus delbrueckii* subsp. *Lactis*[#] *Lactobacillus gallinarum*[#!] *Lactobacillus gasseri*[#] *Lactobacillus helveticus*[#] *Lactobacillus johnsonii*[#] *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens*[#]
Streptococcus	*Streptococcus salivarius* subsp. *thermophilus*
Lactococcus	*Lactococcus Lactis* subsp. *lactis* *Lactococcus cremoris* *Lactococcus Lactis* subsp. *Lactis* biovar diacetylactis
Latilactobacillus	*Latilactobacillus curvatus* [#] *Latilactobacillus sakei*

(continued)

Table 1 (continued)

Genera	Species
Lentilactobacillus	*Lentilactobacillus buchneri* [#!] *Lentilactobacillus hilgardii*[#!] *Lentilactobacillus kefiri* [#!]
Propionibacterium	*Propionibacterium freudenreichii* subsp. *Shermanii*[^,#] *Propionibacterium freudenreichii*[^,#!]
Acidipropionibacterium	*Acidipropionibacterium acidipropionici* [#]
Leuconostoc	*Leuconostoc mesenteroides* subsp. *Mesenteroides*[#] *Leuconostoc citreum*[#!] *Leuconostoc lactis*[#!] *Leuconostoc pseudomesenteroides*[#!]
Levilactobacillus	*Levilactobacillus brevis*[#!]
Ligilactobacillus	*Ligilactobacillus salivarius*[#]
Limosilactobacillus	*Limosilactobacillus fermentum* [^,#] *Limosilactobacillus mucosae* [#!] *Limosilactobacillus panis*[#!] *Limosilactobacillus pontis*[#!] *Limosilactobacillus reuteri* [*,#]
Loigolactobacillus	*Loigolactobacillus coryniformis* [#!]
Mammaliicoccus	*Mammaliicoccus vitulinus*
Oenococcus	*Oenococcus oeni*[#!]
Kluyveromyces[%]	*Kluyveromyces lactis*[#!] *Kluyveromyces marxianus*[#]
Pediococcus	*Pediococcus acidilactici*[#] *Pediococcus pentosaceus*[#]
Staphylococcus	*Staphylococcus xylosus* *Staphylococcus carnosus*
Saccharomyces[%]	*Saccharomyces bayanus*[#!] *Saccharomyces boulardii*[*,#!] *Saccharomyces cerevisiae*[#!] *Saccharomyces pastorianus*[#!]
Schizosaccharomyces[%]	*Schizosaccharomyces pombe*[#!]
Weizmanni	*Weizmannia coagulans*
Xanthophyllomyces[%]	*Xanthophyllomyces dendrorhous*[#!]

*: genera or species available in foods as probiotic (or showing health effect) in USA, symbols marked at species column, separated with other symbols using comma (,)

^: genera or species available in foods as probiotic (or showing health effect) in Australia (includes those that were not authorized by China, which was marked as ^!), symbols marked at species column, separated with other symbols using comma (,).

#: genera or species available in foods as probiotic (or showing health effect) in Canada (includes those that were not authorized by China, which was marked as #!), symbols marked at species column, separated with other symbols using comma (,).

%: yeast, marked at genera column.

Table 2
The approximate composition of various typical probiotic fermented milk products [47, 93–96]

	Yogurt	Kefir	Koumiss	Ymer (Denmark product)	Skyr (Iceland product)
Protein, %	5	3	2.2	5–6	12.7
Fat, %	7.5	0.2	1.9	3.5	0.2
Acidity, %	0.8	1			
Total solids, %	18.5	10.6–14.9		14.5	17.5
Carbohydrate, %		6	2.8	3.5	3.9
Alcohol, %		1	2.2		0.3–0.5
Ash, %		0.7			0.8
Others		1.97 g/L CO_2			

Many fermented milk could contain probiotics, such as yogurt, kefir, koumiss (kumys, kumis, kumiss, coomys), sour cream, and fermented buttermilk. Besides these traditional probiotic fermented milk products, some novel fermented dairy beverages containing probiotics have been developed recently, and the most famous one is Yakult®. The main difference among them is the product status (fluidity) and intrinsic microbial environment (multi vs. single strain) (*see* **Note 3**). They have different substrates, processing procedures, and storage conditions, where the most important is their proximate composition (Table 2). By the time of quality detection, the parameter measured had been regulated by the authorities from various countries. Table 3 summarizes the regulation parameters and numbers of the parameters which the products should achieve.

As mentioned above, the strict definition of probiotic fermented milk should contain live microorganisms in their product matrices. However, recent product development has combined the inactivation of live cells into the processing procedure to extend the shelf life or more stable quality, such as ambient-temperature yogurt (pasteurized fermented milk) and other products. They apply various live-cell inactivation methods to limit or eliminate the activity of viable microorganisms in the products to prolong the shelf life of the products for a farther distribution range or more manageable storage conditions. The inactivation methods include radiation, heating, high pressure, etc. (*see* **Note 4**). There are also coupled designs for these sterilized products about packaging material and style. General packaging uses plastic cups/containers (set) or bottles (stirred) to package fermented milk. For premium products, the glass jar is acceptable to package the product as well.

Table 3
The compilation of fermented milk standards from various countries [27, 38, 97–100]

	Codex Alimentarius	China	USA	Canada	Australia
Fat, %	≤10[acd], 15[b]	3.1[a], 2.5[e]	≥3.25[ab]	——	——
Non-fat-solid, %	——	8.1[a]	≥8.25[ab]	≥9.5[b], 7.6[e], 6.5[f]	——
Protein, %	Min 2.7[abcd]	2.9[a], 2.3[e]	——	≥2.8[b], 2.2[f]	≥3[a] (cow's milk)
Acidity, %	Min 0.3[a], 0.6[bc], 0.7[d]	——	≥0.5[a], 0.7[b] (or ≤ pH 4.6)	≥0.7[b]	≤pH 4.5[a]
Acidity, °T	——	70	——	——	——
Microbial load, cfu/g(mL)	Min 10^{6} [ab] (total), 10^{4} [cd] (yeast)	≥10^{6} [ae]	≥10^{7} [a]	≥10^{7} [b]	≥10^{6} [a]
Ethanol, %vol./w	Min 0.5[d]	——	——	——	——
Document number	CXS 243–2003	GB19302–2010	FDA-21 CFR 131.112, 131.200	National Dairy Code, Part III	Australia New Zealand Food Standards 2.5.3, F2015L00413

[a]Fermented milk/Cultured milk.
[b]Yogurt, alternate culture yogurt, acidophilus milk (Yogurt: fermented milk using *Lactobacillus bulgaricus* and *Streptococcus thermophilus* as culture; alternate culture yogurt: using *Streptococcus thermophilus* and any *Lactobacillus* species; acidophilus milk: using *Lactobacillus acidophilus* as culture).
[c]Kefir.
[d]Kumys.
[e]Flavored fermented milk (with sugar or fruit component addition).
[f]Yogurt drink (drinkable fermented milk).
——: Not mentioned or required by such standards.

However, novel tetra packaging was developed to comply with the requirement of ambient-temperature fermented milk to assist its prolonged storage time. The shelf life of regular fermented milk (with or without probiotics) is around 21–28 days. For plastic packaged products, some of them can be shortened to 14 days (it is worth noting that the shelf life does not have a severe relationship with the preservation ability of LAB or the health effect of probiotics). The optimal storage condition of such products is around 4 °C, requiring fully cold-chain logistics. For ambient-temperature fermented milk, the shelf life can be extended to 6 months at ambient temperature (around 25 °C).

Moreover, there are vast amounts of products commercially available in the market. Still, they can be characterized according to several criteria, such as matrix status (set/stirred), product additive (natural, sweetened (flavored), nutritionally enhanced), post-fermentation processing (condensed, frozen, carbonized, spray-dried), fat content (full-fat, partially skimmed, skimmed, and Greek yogurt) [11]. Nevertheless, their material, main processing procedure, and packaging step are very similar, with a slight difference in additive, post-fermentation, and packaging steps. These will be described in detail in **Part III**.

2 Material

Materials used for probiotic fermented milk production can be divided into several groups: raw milk and milk substrate, starter culture and probiotic strains, sweetener and additive. They have different effects on the probiotic fermented milk, which should be focused on during processing.

2.1 Raw Milk and Milk Substrate

The substrate and primary material of probiotic fermented milk should be various milk originating from multiple breeds or species of mammals. Commonly seen dairy animal species include cows, goats, sheep, buffalo, donkeys, and camels, where cows are the most used for raw milk production. Bovine milk is the most consumed milk by humans. Various cattle breeds have been domesticated by humans for milk production (some of the breeds are for both milk and beef). These temperate breeds include Ayrshire, Guernsey, Brown Swiss, Shorthorn, Jersey, and Holstein Friesian. Among them, Holstein Friesian is the only most important breed for milk production. Holstein Friesian originated from the Netherlands and had been exported widely to the world due to its adaptability. It has excellent milk production capability, where its average milk yield is 25–35 kg/day [11]. This yield is far from other dairy breeds. Holstein Friesian has a lower milk fat content than other temperate breeds except for Shorthorn [11]. The typical appearance of Holstein Friesian is black and white color. Besides, other

species have their characteristics, such as Jersey has a high milk fat (4.95%) content and dry matter (14.54%) content with low yield (19–25 kg/day), and shorthorn has a high protein-fat ratio but low yield as well (17–25 kg/day) [11]. Therefore, the selection of raw milk sources would significantly affect the final product's quality.

Notably, the quality of raw milk produced by different animals can be affected by various factors. Of which the most important and controllable are milking season, feeding (water and fodder), and equipment. The raw milk composition could be varied significantly following the milking season (lactation season) change, but the lactose in the milk could be stable. Protein and milk fat have a solid response to season change, where the lowest content occurred in summer (3.21% for protein, 4.1% for fat) and the highest content occurred in winter (3.38% for protein, 4.57% for fat), respectively [12]. It had been reported that the raw milk yield and composition were negatively related to environmental temperature [13–17]. This phenomenon is reasonable and explainable due to the Holstein Friesian originating from a cool area, which has a stress reaction to heat. Heat stress is one of the most significant issues in cows, especially Holstein Friesian husbandry. Lactation season could also affect raw milk yield and composition, whereas Holstein Friesian's lactation season could be over 200 days. Raw milk yield and composition have fluctuated over a long period [17]. The raw milk yield increases and reaches a peak during the early lactation period but goes lower following the lactation period [17]. The fat content has a real controversial tendency compared with yield [17]. It went lower at the beginning of the lactation period and turned to increase, accompanied by lactation progress [17]. Milk protein also has higher content at the beginning of the lactation period [17].

Water and forage feed could be crucial factors that impact the raw milk quality, where the contaminant and odor components, such as heavy metals, animal drugs, and toxins, could be transmitted to the milk through cow's milk secretion [18]. The type and quality of forage could also affect the milk fat content and composition, where the involvement of phytochemical composition in the forage attracts attention [19]. The feeding method could influence the quality of raw milk as well. Grazing cows have lower raw milk yield than feedlot cows, but the fat content in grazing cows' milk is higher than in feedlot cows' milk. The difference between the protein content is negligible [18, 20]. It is worth noting that the fatty acid composition in the milk produced from grazing or feedlot cow is also different. In summary, it is wise to determine the source of raw milk regarding the abovementioned factors before adopting it in fermented milk production for better product quality.

Apart from species, breed, and lactation season, and feeding material quality and method, milking sanitation and equipment are also critical to raw milk quality, especially microbial load. Essential

sanitation of the cows' udder (or other dairy animals) is necessary as the microbe in the raw milk strongly correlates with teat skin sanitation. Research proved that the microbial composition is significantly different between raw milk and teat skin due to the both-way contamination. However, 92.1% of the bacteria in the raw milk come from the teats' skin (genetically connected) [21]. An efficient way to sanitize the udder is teat dipping (pre and post), in which the teat was sanitized via iodine solution. The same research also revealed that the microbial composition of teat skin is significantly similar to raw milk, which means the both-way contaminations were halted, and the microbial was only transferred from raw milk to teat [21]. This result proved that iodine sanitation is an efficient way to intercept teat-raw milk contamination. Sanitation of milk equipment is also a pivotal step in ensuring the quality of raw milk. Research indicates that appropriate sanitized equipment could reduce raw milk's thermophilic spore load [22]. Other factors that have relationships with low spore load include farming environment, husbandry scale, regular udder massage, and others [22]. These factors also confirmed that appropriate farming methods, feeding (fodder and silage), housing conditions, and even the cow's mood influence the raw milk quality, which needs attention.

Milking is an essential step for raw milk collection, where the equipment evolution has served this step well. Machine milking has far higher efficiency than manual milking, which has improved the raw milk yield significantly [11]. Recently, automatic milking equipment (robotic milking) was developed to avoid excess stress on cows and save human labor. This equipment ensures the animal welfare of cows and eases their nervousness, anxiety or other negative moods to prevent low-quality raw milk. Usually, the cows were tagged and managed via ear tag, where the information of each cow can be collected when they enter the milking robot for milk tracing. The cost of milking also decreased compared with traditional milking. This automated milking machine has attracted the attention of farmers from developed countries, such as the USA, Australia, The Netherlands, and New Zealand, to apply this system for better raw milk production.

After milking, the raw milk should be tested before production. Some standards or codes require the quality of raw milk. The most crucial parameters are microbial load and somatic cell count (SCC). For microbial load, the USA requires that the raw milk for direct consumption should not contain more than 15,000 total viable bacteria/mL and < 10 coliform bacteria/mL [23]; China has a 2×10^6/mL total viable microbial count limitation of raw milk, whereas the EU limited the total viable microbial count to 1×10^6/mL [24]. For somatic cells, it is not required by China, but the USA and EU had limited the count below 6×10^6 and 4×10^6 cells/mL, respectively [23, 25]. Somatic cell count (SCC) is vital for raw milk quality. It indicates the health status of cows or other dairy animals.

SCC was influenced by parities, calving season, and lactation period, and the yield will drop dramatically when the SCC goes higher [26]. Research proved that the composition of raw milk reached the lowest amount when the SCC exceeded 5×10^6/mL; hence the researcher recommended that the SCC in raw milk should not be above 5×10^6 cell/mL [26].

Besides the microbial count and SCC, many other parameters should be satisfied, including fat, protein, and non-fat milk solids in many countries. For industry raw milk collection, many essential tests need to be performed to ensure the quality of raw milk and perspective products. These include sensory tests, ethanol tests, clot-on-boil tests, titratable acidity, density test, microbial (dye reduction methods)/somatic cell/antibiotic test, composition determination, and adulterant tests [11]. Among them, the ethanol test is a rapid method to determine whether the raw milk is fresh or not, based on the acidity of raw milk [11]. This is a very fast and easy method to be applied in the industry due to the simple phenomenon, equipment, and indicative capability. For fresh raw milk, there will be no phenomenon when ethanol (68%, 70%, 72%) is added to the raw milk, where the coagulation of casein (protein) will occur when the raw milk deteriorates [11, 18]. Notably, a microbial/somatic cell/antibiotic test is necessary, especially for fermented milk production. Besides the microbial count, excess antibiotic in the raw milk is crucial for fermented milk production due to their inhibitory effect on the starter culture cultivation and growth, especially probiotic, which requires a strict environment. The source of antibiotics is vast, but it may come from cattle disease treatment, fodder additive residue, and milking contamination [18]. Addition of antibiotics purposely is rarely seen, but it affects the quality significantly, which needs strict regulation. Developed countries require that antibiotics should not be tested in raw milk [18]. However, a trace amount of antibiotics is still allowed in developing countries [27], indicating that raw milk should be appropriately tested and treated when applied to produce fermented milk in these countries.

After collection, pre-treatment should be performed to ensure the quality of raw milk for further production. Usually, pre-treatment includes filtration, purification, cooling, pre-pasteurization (optional), and deaeration (optional) [11]. Filtration and purification could efficiently remove physical contaminants and excessive microbial and somatic cells to reduce observable contaminants by the naked eye. However, rapid cooling is essential for the stable quality of raw milk during storage before processing. Usually, freshly collected milk has cow's body temperature, which should be cooled around 4–6 °C as soon as possible. The growth of microbes could be attenuated or inhibited at this temperature. If its temperature could be cooled down to 2–3 °C, the growth of the microorganism could be near completely halted,

and it can be stored for about 7 days [11]. Pre-pasteurization should be performed if the raw milk is not used immediately to avoid quality deterioration.

2.2 Starter Culture and Probiotic Strains

Starter culture is essential for probiotic fermented milk production. It usually contained lactic acid and polysaccharides producers, such as *Lactobacillus* (L) and *Streptococcus* spp. (S). The ratio of L/S is around 1:1 or 1:2, where the overwhelming of *Lactobacillus* will result in excess lactic acid content and unacceptable flavor [11]. Detailed starter culture production will not be mentioned here. Still, the type of starter culture and production of starter culture are described in Table 4 and Fig. 1, respectively. It is worth noting that adding probiotics as a starter culture is the main method to incorporate probiotics into fermented milk. Hence, the cultivation of probiotics needs further attention. The synergistic or antagonistic bio-relationship between conventional starter culture (*Lactobacillus & Streptococcus* (L&S)) and probiotic could affect the success of fermentation [28]. For example, the difference between the growth rate of L&S and probiotic leads to desired microorganism cultivation failure, or the metabolites of each species could promote or inhibit the growth of other species (hydrogen peroxide, oxygen content, carbonized, etc.) [28–34].

Table 4
Various types of starter cultures used in fermented milk production [11, 40, 47]

Classification criteria	Type of starter culture	Notes
Preparation of Starter cultures	LAB pure culture	Primary strains included in the culture (step 1)
	Mother starter culture	Proliferation of primary strains (step 2)
	Bulk starter culture	Proliferation of mother culture, used for manufacture directly (step 3)
Strain composition of Starter cultures	Mixed strains starter culture	Contains more than one strains for synergistic fermentation
	Single strain starter culture	Contains only one strain, mixed when applying
	Supplemented strains starter culture	Contains one or more strains for special purpose, includes exopolysaccharides production, aroma component production, and probiotics
Status of Starter culture	Liquid starter culture	Easy to operate and cheap, but the viability can be weakened
	Powder starter culture	Better viability and stability than liquid form
	Frozen starter culture	Highly concentrated, highest viability, direct usage

LAB lactic acid bacteria
Steps 1, 2, 3: The steps required for starter culture application during production procedure. These steps were performed according to factories in situ application.

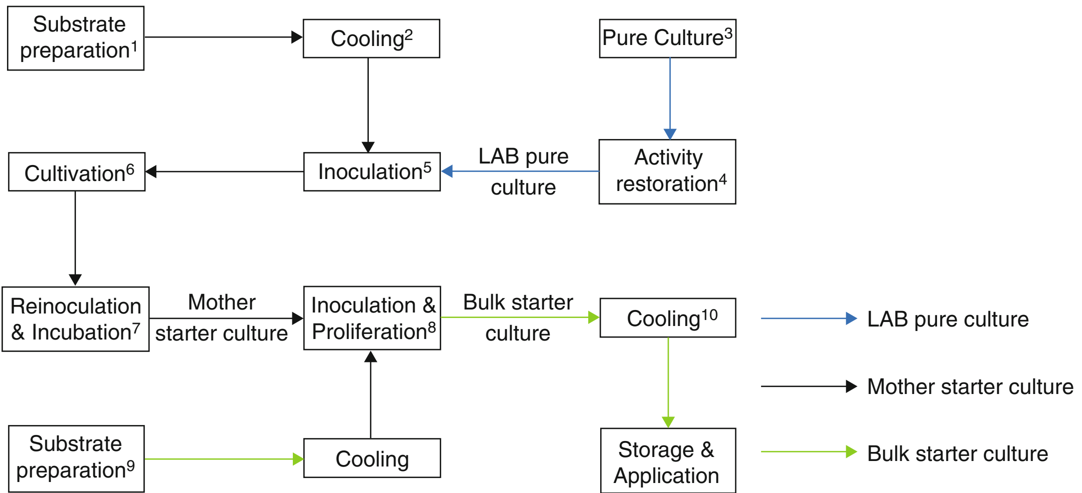

Fig. 1 The flow chart of starter culture preparation [11, 40]. (1) Reconstituted skimmed milk (10–12% solid), heated 90–95 °C for 30–40 min or 121 °C for 15 min. (2) Mesophilic culture: 20–30 °C; theromophilic culture: 42–45 °C. (3) 0–4 °C storage, subculture every 1–2 weeks; random purification is needed. (4) Restoration for 2–3 times. (5) 1–2% addition amount. (6) Temperature determination according to strain characteristics; Time: 3–20 h. (7) Same condition or 2–3 times. (8) At 42 °C, stop when acidity $>$0.8%. (9) Same substrate treatment condition, but using product raw material as substrate, 1–2% of total raw material. (10) Use within 6 h: 10–20 °C; use after 6 h: 4–5 °C

Due to the growth rate, the time and form of probiotic addition are crucial. As for the preservation of the viability of probiotics, many ways are used to protect probiotics and assist them in reaching the GI tract without severe weakening due to lactic acid in fermented milk or harsh condition in the GI tract. Encapsulation is a commonly used method to protect probiotics. Probiotics can be encapsulated (usually microencapsulated) in different wall materials or matrices to maintain viability (*see* Chap. 14 for more details). Different wall material has various properties, such as protection, lyse, texture alteration, etc. There is a study that showed that the addition of microencapsulated probiotics could affect the texture of yogurt (smoothness), which needs attention (alginate-starch as wall material, which can affect the texture) [35]. Other materials used for microencapsulation include whey protein (an useful by-product of cheese production), gellan gum (polysaccharides), etc. The microencapsulation method includes drop-out, emulsification, extrusion, coacervation, and others. Compared with extrusion, emulsification has a higher encapsulation rate [36]. Microencapsulated probiotics can shorten the fermentation time of fermented milk as well [36], but this phenomenon needs further clarification to differentiate between bacteria synergistic effect or microencapsulation promotion. Besides, the strong buffer capacity of the substrate (neutralized pH) or the firm texture of fermented milk (gel) (prevents acid contact with probiotics) can protect probiotics efficiently as well [28].

2.3 Sweetener and Additives

Many additives can be used in probiotic fermented milk, where the sweetener is the most important one. Sweeteners could provide a sweet taste to the consumer to assimilate or cover the harsh taste of lactic acid in the fermented milk. A commonly used sweetener is sugar (sucrose), which is accepted by most consumers. Recently, artificial sweeteners, such as sucralose and aspartame, were used to provide a more intense sweet taste and reduce cost. However, the health requirement of customers had forced the producer to replace artificial sweeteners with natural sweeteners, hence stevia, erythritol, and mogroside have come into sight of the producers. These selections have broadened the horizon of sweeteners from a health perspective and increased the acceptability and functionality of fermented milk. Besides, there are other additives, such as fruit components (jam, crushed or pulp), thickener/stabilizer/emulsifier, essence, pigment/colorant, etc. [11], that can be added into the fermented milk in accordance with local regulations.

It is worth noting that some unique carbohydrates, such as dietary fiber, resistance starch, oligosaccharides, and inulin, were added to the probiotic fermented milk to acquire its health benefit and probiotic promoting capability (synbiotic ability). These substances are called as prebiotics. Prebiotics is a type of food component that could not be digested by the endogenous host enzymes yet could exert benefit on the host by modulating gut microflora [37]. In this case, the type, purity, chain length, percentage of prebiotic, target probiotic/microflora, product formula and characteristic, and storage conditions need to be considered when applying prebiotic in probiotic fermented milk [37]. Prebiotics can significantly affect the probiotic viability and the physiochemical (texture and rheology), organoleptic and functional properties of the products [37]. However, the effect (positive, negative, or neutral) is still under debate, which needs more attention when utilizing them in the products [37]. More detailed availably of thickener (thickening technology) and prebiotic selection will be discussed in **Notes 5** and **6**.

3 Method

The production method of probiotic fermented milk is similar to yogurt production, which involves pre-treatment (standardization, pre-heating, homogenization, heating, cooling), inoculation, fermentation, additive addition, and packaging. The flow chart of the processing procedure is shown in Fig. 2. Here, it is notable that the order of fermentation, packaging, and additive use is different between set-fermented milk and stirred-fermented milk. Detailed order is shown in Fig. 2 as well. In the following paragraph, each step will be discussed separately, and the combination of such steps should be performed as per product and in situ requirements.

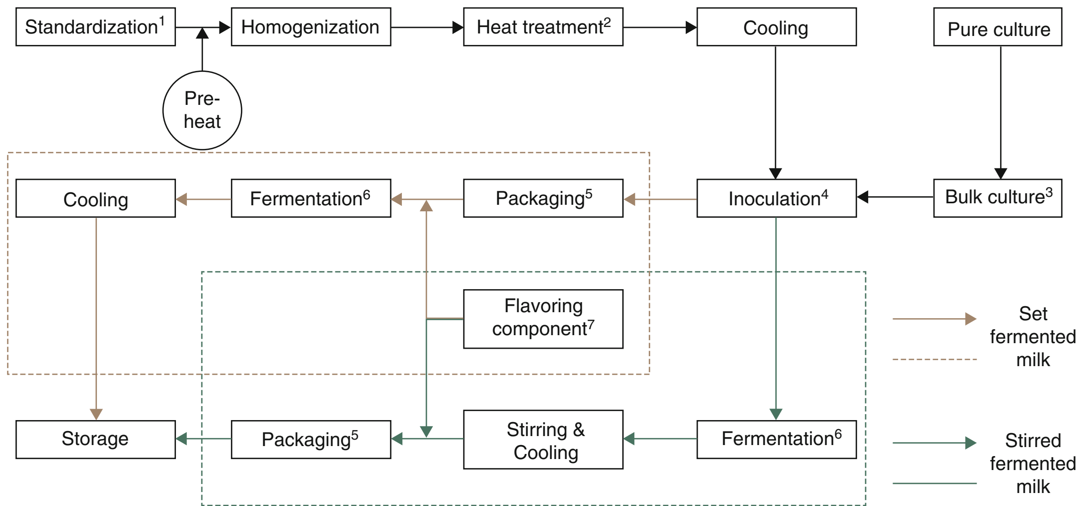

Fig. 2 The flow chart of fermented milk processing [11, 40, 47]. (1) Milk solid, includes protein, cream, thickener, sweeteners were added here; filtration may be applicable for unsolved substances, critical control point 2 (CCP 2) for both set and stirred fermented milk. (2) Significantly important for product quality control, CCP3 for both set and stirred fermented milk. (3) The hygienic condition of starter culture is important, CCP4 for both set and stirred fermented milk. (4) The control of hygienic condition and relative parameter is critical for this step, CCP5 for both set and stirred fermented milk. (5) The hygienic condition of environment and packaging container is critical, CCP6 for set fermented milk, CCP 7 for stirred fermented milk. (6) The fermentation temperature and time are critical for the success of products processing, CCP 7 for set-fermented milk, CCP 6 for stirred fermented milk. (7) Includes fruit component (pulp or jam), essence substances, etc

3.1 Pre-Treatment

Pre-treatment includes raw milk standardization, homogenization, heat treatment, and inoculation steps. Firstly, the raw milk pumped from the storage tank should be standardized to fulfill the requirement of local regulations where the factory resides, or the product will sell. In general, any product should satisfy the requirement of FAO/WHO regulation [38] for global distribution and retail selling. Fat and protein content should be less than 10% and more than 2.7%, respectively. Hence, any raw milk that does not meet this requirement should be standardized to achieve this limitation. Usually, the fermented milk fat content is between 0.5–3.0% [11], depending on whether it is skim or not, where the addition of cream is necessary to adjust this content to not only fulfill the regulation but also to guarantee the sensation of such product. Besides, the non-fat-solid of milk will be fortified, if necessary, whereas the skimmed milk powder should be used here. These components (cream, skimmed milk powder) can be provided within the factory from other product lines to utilize the by-product and make the best value of it. The sugar and stabilizer should be added here to favor the growth and fermentation of

starter culture and possess desired texture of the product. Detailed additive addition will be discussed in the Subheading 3.4.

After standardization, milk should be pumped into the heater to pre-heat for homogenization. Appropriate heating could stabilize the fat globule in the milk for homogenization in case any undesired consequences occurred, such as fat separation (creaming) or incomplete homogenization. Homogenization aims to shrink the size of fat globules to prevent cream separation and unify fat distribution. Hence, the stability and consistency of the fermented milk could be improved. Further, this step could mix the ingredients added during standardization well to enhance the texture and mouthfeel of the final product [11, 39]. This step does not affect the growth of probiotics but could increase viable cell count [39]. In general, appropriate pressure should be 20–25 MPa at 60–65 °C [11]. However, slight modification should be applied in accordance with in situ, such as higher pressure for a higher amount of stabilizer or thickener. The time for homogenization varied significantly, which depends on the volume of milk to be homogenized.

The homogenization will not reduce the temperature of milk significantly, where it should be followed by further heating to sterilize the milk for fermentation. Any living microorganism will be killed during this step, but the spore may not be eliminated due to its heat resistance. However, the fermentation and growth of starter culture (including probiotic) could occupy the niche for spore growth, which make the product consumable. Meanwhile, heating could inactivate intrinsic antimicrobial components, such as some antimicrobial peptides or proteins, to favor the growth of starter culture [11]. Further, heating could denature whey protein to modify its tissue to improve viscosity and prevent whey separation [11]. Usually, an appropriate heating condition should be 90–95 °C for 5 min [40], where 120 °C for 3–5 s is acceptable, such as in Ultra High Temperature processed milk (UHT).

Scalding milk should be gradually and immediately cooled to ~40 °C for inoculation. Traditionally, yogurt fermentation uses 43 °C for fermentation, with a starter culture addition of 2–4% [11, 40]. However, this temperature should be modified when probiotics are incorporated into the starter culture for fermentation. The synergistic and antagonistic effect among bacteria or yeasts should be considered to obtain the best probiotic growth with acceptable product quality. Mostly, the optimal growth condition of probiotics is around 37 °C for many genera, such as *Lactobacilli*, *Bifidobacteria* [41], and the optimal condition of *Propionibacterium* is around 30 °C [42]. However, the starter culture bacteria (*Lactobacillus delbrueckii* subsp. *Bulgaricus*, and *Streptococcus thermophilus*) perform badly under this condition (lower lactic acid, volatile component, polysaccharides production, etc.), hence appropriate fermentation temperature modification

should be determined previously during the pilot plant test before larger scale production, as well as the amount of addition, if the starter culture was developed by the fermented milk producer itself. Otherwise, sticking to starter culture instruction provided by the starter culture manufacturer (if applicable) is a wise decision to guarantee the success of fermentation. Previous thorough agitation is recommended for starter culture before addition for better performance [11]. Notably, the sterile operation is crucial for this step due to processing demand. There will be no more sterilization or pasteurization involved (generally, but there is ambient-temperature fermented milk available in the market, which is discussed in **Note 4**), where any contaminant (bacteria, yeast, mold, bacteriophage, etc.) introduced into the product will affect the quality of probiotic fermented milk significantly, hence causing severe consequences or results. This step and fruit pulp or jam addition (discussed in Subheading 3.4) are both critical control points of fermented milk production, which requires complete and careful administration.

3.2 Fermentation

Fermentation is the most critical step of fermented milk processing to obtain desired flavor and texture. The order of fermentation and packaging is decided by the desired fermented milk texture (set, stirred, or drinking). Here, we discussed fermentation firstly, then packaging, but the order can be changed. In general, starter culture contains *Lactobacillus delbrueckii* subsp. *bulgaricus* (L) and *Streptococcus thermophilus* (S) and requires a fermentation temperature around 41–42 °C for 2.5–4.0 h fermentation time (2–4% addition) [11]. However, introducing probiotics into the starter culture altered the appropriate fermentation temperature. As mentioned above, probiotic strains have the best performance when the temperature reaches 37 °C, but L&S cannot grow well at this temperature. Even the antimicrobial properties of probiotics could inhibit the growth of L&S, and the fermentation fails. Also, probiotic requires a longer fermentation time, from 8–9 h to 48 h, even some requires 72 h [42–45], this had led to a more difficult determination of fermentation time. Hence, an appropriate adjustment should be performed for fermentation conditions to facilitate the growth of both L&S and probiotic. For example, two-step fermentation is a practical way to ferment milk containing complicated microbial environments, such as kefir. Yoo et al. [46] developed a two-step fermentation method, which applied 37 °C for 9 h at the first step, and then 24 °C for 15 h for the second step. This method had acquired better sensory acceptance. Therefore, appropriate adjustment or separation of such fermented time or temperature could be applied to fit the growth of all the strains. Some probiotic strains can also grow at 40 °C, which is strain-specific, but this could also provide chances for producers to ferment milk at this temperature.

For set-fermented milk, the milk and starter culture mixture are packaged into the container firstly, which is plastic or glass, but the hygienic and aseptic conditions should be guaranteed before packaging. The packaged (sealed) products are placed in a warm room (fermentation room) which has appropriate spaces between the containers for better airflow [11, 40]. Stable temperature and shaking avoidance should be monitored during the whole procedure in case tissue breakdown or fermentation quality deteriorates [47]. The fermentation should be stopped when pH arrived at 4.6 and appropriate curdling happen in the container. At this time, immediate cooling is essential for controlling the acid content in the fermented milk. Generally, the temperature should be cooled down to 35 °C within 30 min, then 18–20 °C in the next 30–40 min, then 5 °C as soon as possible [11] and wait for distribution.

For stirred fermented milk, this fermentation step is carried out in a fermentation tank, which requires uniform temperature distribution in the tank due to the tank size. The upper and lower tank temperature difference should not exceed 1.5 °C [11]. When the fermentation is stopped (pH 4.2–4.5), immediate cooling is required to avoid excess acid production or flavor deterioration (*see* **Note 7**). However, cooling down should not be too fast, which could lead to curd shrink, and whey may be squeezed out of the curd [11]. The stirred fermented milk is agitated, in which mechanical force is involved in agitation. Appropriate control of such process is needed to maintain the tissue structure. Slow stirring, medium stirring temperature (10–25 °C), and desired pH (below 4.7) should be affirmed to maintain the tissue structure and avoid whey isolation due to mechanical force [11, 40]. Mechanical force may also occur when pumping the fermented milk for transportation due to turbulence, so slow pumping is needed to prevent any undesired results. The flow rate should be maintained below 0.5 m/s [11]. However, any parameters mentioned here are adjustable in accordance with the actual textural and other sensory properties of fermented milk products, where the pilot test is significantly essential to obtain the best parameter to favor the production.

3.3 Packaging and Storage

Packaging material can be plastic or glass, with different container shapes. Bottles, cups, bowls, or jars are all acceptable. It depends on the product or consumer demand. For set-fermented milk, the milk and starter culture mixture is packaged into retail containers/cups, with or without additives, to prepare for fermentation. However, for stirred-fermented milk, the fermented milk is packaged at 15–22 °C when mixed with additive or not [11, 40]. It is significantly vital to ensure aseptic and sterile condition during any packaging step, especially air cleanness. This is the rare step where products are exposed to and have contact with the outer environment, hence complete cleanness needs to be focused on to avoid introducing contaminants into the products.

Besides traditional packaging, the novel product pushed the development of packaging. There is a pasteurized fermented milk product commercially available in China, which uses Tetra Prisma® Aseptic to ensure the shelf life (6 months) (*see* Fig. 3). Other types

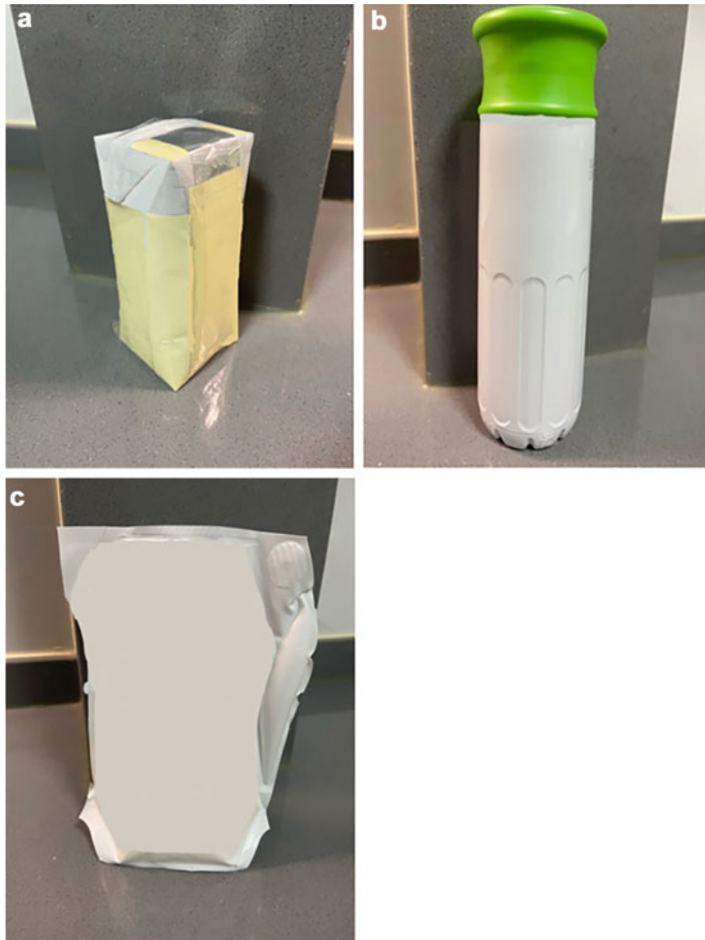

Fig. 3 (**a**) The photos of Tetra Prisma® Aseptic package, (**b**) plastic bottle for ambient-temperature fermented milk, and (**c**) Ecolean® package. *optional. (1) Skimmed milk powder is dissolved at 50–55 °C warm water where sterilization of such substrate is optional. (2) *Lactobacillus casei* Shirota as the culture seed was added into the substrate for incubation at 37 °C, and stopped when appropriate parameters were detected. (3) Culture base (fermented substrate) were stored at 5 °C after sweetened by syrup. (4): The sweetened culture base was mixed with sterilized water for better fluidity. (5): Bottles were made by food-grade polystyrene and transported with clean air for following selection step. Bottle selector makes the bottle oriented to the same direction for decorating (printing). The logo was printed on the bottle using instant-dry red ink (for sugar-reduced version, it is blue ink with more complicated decoration). (6) The content of each Yakult bottle is 100 mL, and the cap was made by aluminum foil which is easily opened. (7) The product was stored at 5 °C for following distribution, but it should be maintained at this temperature when selling and at home until consumption

of packaging are also available for this particular product, such as plastic bottles, which are rarely seen in traditional fermented milk packaging. Ecolean® air is also an available packaging for fresh stirred fermented milk due to its low weight, unique handle (air-filled), and suitability for straw use. The detailed production method of ambient-temperature fermented milk will be discussed in **Note 4**.

The storage condition of fermented milk products should be around 4–5 °C, where the storage step begins after packaging (stirred) or fermentation (set), depending on their production procedure. Usually, the storage time (shelf life) for traditionally fermented milk (yogurt) is around 28 days at 5 °C. However, research revealed that the *Lactobacillus acidophilus* could drop significantly after 21 days of storage at 5 °C, where *Lacticaseibacillus casei* have the highest viability retention [48]. These results indicate the incorporation of probiotics should be considered when examining shelf life to maintain the essential viability of probiotics.

3.4 Additive Addition

There are many additives available for fermented milk production. Common additives include fruit flavor components (mainly pulp or jam), sweetener, thickener/stabilizer, or other flavor ingredients, such as nuts or raisins. Detailed additive selection and commercially used novel additives will be discussed in **Notes 4–6**. Here, the procedure operation will be mainly introduced. As mentioned above, the milk-solid enhancers (protein, fat, etc.) are added at the beginning of production at the pre-treatment stage [11, 40, 47]. Protein is usually stored and sold as solid statues, where it needs to be added and solved into milk. Appropriate agitation is important to maintain the uniform milk texture and nutritious component distribution in the milk. Conversely, milk fat is usually added in liquid form (milk cream), which does not require long-time agitation. Excessive agitation or stirring would isolate fat and induce quality deterioration, such as unpleasant mouth sensation or lack of aroma. Sugars and other stabilizers are added at this stage for a better solution. Sugar (sucrose) is essential for certain microbial growth as well, making it a pivotal component to favor the growth of probiotics, especially for those non-lactose fermenters.

In general, fruit components or other flavor ingredients are added just before packaging [47] due to heat treatment could cause unexpected fouling in pipe or component degradation. However, this raised the hygiene issue when adding these ingredients. There is no more heat treatment following this addition, and the possibility of introducing contaminants needs to be controlled. As mentioned above, this is a critical control point of the whole processing procedure. Hence, complete sterilization of such ingredients should be guaranteed to ensure the safety and quality of desired products.

4 Notes

1. Several probiotics can be applied as starter cultures, where the commonly used probiotic species are listed in Table 5. It should be noted that the health effect of probiotics is strain-specific, hence the claim of strains on the label is necessary for legal compliance. However, as mentioned above, there is no precise list of probiotics in most countries, therefore a thorough evaluation of probiotics needs to be performed, especially for novel probiotic strains to ensure the availability of the strains. China has published a new probiotic standard (*Probiotic Food*, T/CNFIA 131–2021) which gives a good indication of the evaluation procedure applied to probiotic food. This evaluation

Table 5
Predominantly used thickeners/stabilizers, probiotics, prebiotics, and sweeteners in fermented milk products at present

Additive type	Name of the additive	Additive type	Name of the additive
Thickener	Acetylated distarch adipate	Sweetener	Acesulfame potassium
	Acetylated distarch phosphate[a]		Aspartame
	Agar[a]		Erythritol[a]
	Carob bean gum		Isomaltose (palatinose)
	Diacetyl tartaric acid ester of mono (di) glycerides (DATEM)		Jam[b]
	Gelatin		Lactase[b]
	Gellan gum[a]		Maltitol & maltitol syrup
	Guar gum		Mogroside
	Hydroxypropyl distarch phosphate		Neotame
	Lactic and fatty acid esters of glycerol		Steviol glycosides[a]
	Pectin[a]		Sucralose
	Starch		Sucrose[a]
			Sweetened condensed milk[b]
Probiotics	*Bifidobacterium*		Xylitol
	Lacticaseibacillus paracasei (Formerly *Lactobacillus paracasei*)		
	Lacticaseibacillus rhamnosus (Formerly *Lactobacillus rhamnosus*)	Prebiotic	Plant powder
	Lactobacillus acidophilus		Inulin
	Lactobacillus delbrueckii subsp. *Bulgaricus* (*Lactobacillus bulgaricus*)		Polydextrose
	Lactococcus Lactis subsp. Cremoris		Resistance dextrin
	Lactococcus Lactis subsp. *Diacetylactis*		Fructo-oligosaccharides
	Lactococcus Lactis subsp. Lactis		
	Leuconostoc mesenteroides subsp. *Mesenteroides*		
	Streptococcus thermophilus		

[a]could be used in ambient-temperature fermented milk
[b]the additive can provide sweetness but not a sweet additive (sweetener)

includes (1) probiotic species and strain identification with clear and well-known sources, (2) probiotic preservation method and safety evaluation, (3) whole-genome sequencing, which is peer-reviewed, and (4) in vivo or randomized clinical trial which supports its health effect [9].

2. Probiotic fermented milk has the potential to be claimed as food for a special purpose, but appropriate legal appliance and related clinical trial needs to be performed. For example, foods for health purposes need to be registered in China, and clearly label the registration number on the packaging bottle or other forms of packaging. Other countries have their regulations, where the individual analysis of local law, regulation, and policy is significantly vital for such claims. It should be mentioned that some products claim to provide nutrients to >3-year kids, but they are normal foods instead of foods for special purposes. The targeted population of the products limits the type of such product, which needs to be considered when developing products.
3. Dairy-based probiotic fermented beverage (probiotic beverage) is an alternative product that contains live probiotics but is more drinkable than traditional fermented milk. Yakult® is a popular product that is a representative of such products. It has a special and patented probiotic, *Lacticaseibacillus casei* Shirota, which was isolated by Minoru Shirota in 1930. The phenotype of this strain is similar to other *Lactobacillus* spp., and it is worth noting that it could grow at temperatures 15 °C–41 °C, but the optimal temperature is 37 °C [49], as mentioned above. *Lacticaseibacillus casei* Shirota is a sucrose fermenter [49], where possible sugar addition may involve in the production procedure, just before fermentation for better lactic acid production, but this depends on in situ application, not compulsory. The brief manufacturing procedure includes milk reconstitution, sterilization, fermentation, homogenization, flavoring, balancing (adding sterilized water to dilute the concentrated product), packaging, and further storage [49] (*see* Fig. 4). The uniqueness here is that probiotic beverages use skimmed milk powder to reconstituted milk as their fermentation substrate to minimize the effect of milk fat (fat isolation, as mentioned in **Note 6**) and control cost. Moreover, the sterilization procedure conferred brown color to the substrate, just like roasted fermented milk (*see* **Note 4**), due to such high temperature and time. Meanwhile, the fermentation temperature is maintained at 37 °C for better growth of the *Lacticaseibacillus casei* Shirota. This temperature differs from traditional fermented milk due to the simplified microbial environment (multi strains vs. individual strain).

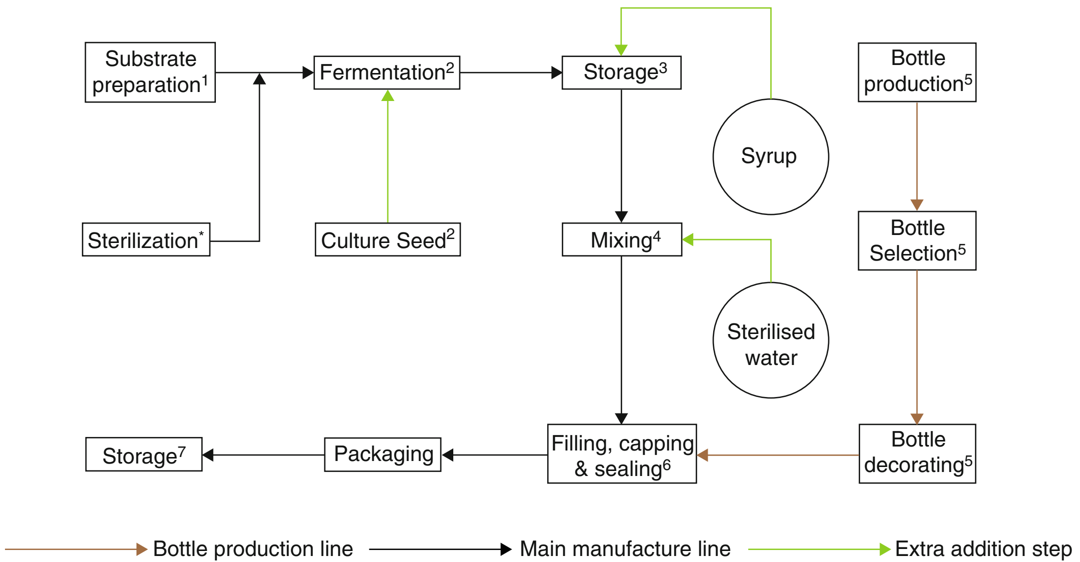

Fig. 4 The manufacturing flow chart of Yakult® [49]. *: optional (1) Skim milk powder is dissolved at 50–55 °C warm water where sterilisation of such substrate is optional. (2) Lactobacillus casei Shirota as the culture seed was added into the substrate for incubation at 37 °C, and stopped when appropriate parameters were detected. (3) Culture base (fermented substrate) were stored at 5 °C after sweetened by syrup. (4) The sweetened culture base was mixed with sterilised water for better fluidity. (5) Bottles were made by food-grade polystyrene and transported with clean air for following selection step. Bottle selector makes the bottle oriented to the same direction for decorating (printing). The logo was printed to the bottle using instant-dry red ink (for sugar-reduced version, it is blue ink with more complicated decoration). (6) The content of each Yakult bottle is 100 mL, and the cap was made by aluminum foil which is easily opened. (7) The product was stored at 5for following distribution, but it should be maintained at this temperature when selling and home storage until consumption

Additionally, balancing is a particular step for probiotic beverage production. This step added sterilized water into the product to dilute it and confer higher fluidity, making it more drinkable to mimic beverage status. This step differentiated Yakult® from traditional fermented milk products. Interestingly, the bottle of Yakult® is produced at the same factory by molding food-grade polystyrene [49]. This design made the packaging bottle controllable, but the Yakult® product should be stored avoiding light and kept at 4 °C due to the semi-transparent properties of the bottle. Other probiotic beverages have a very similar procedure, where the difference is the strain used. *Lacticaseibacillus paracasei* is the most used species for other probiotic beverages. However, the commercialization potential of *Lacticaseibacillus casei* Zhang is growing and has performed good ability to be utilized in yogurt and some health effect on rats [50–52].

4. As mentioned above, some unique products are commercially available in China with high sales. One of the most popular

products is pasteurized or sterilized fermented milk. It is also called ambient-temperature fermented milk, which indicates its most valued characteristic. It can be stored under ambient temperature with no quality deterioration for 6 months. The unusual step involved for this long storage is the following sterilization or pasteurization after fermentation [53, 54]. This step killed all available live microorganisms in the fermented milk, which stopped continuing fermentation usually occurring in unsterilized or unpasteurized fermented milk (traditional fermented milk). The pasteurization or sterilization method occurs by heating (72–121 °C, 4 s–20 min, usually 75 °C, 20 min) and high pressure (600–680 MPa, 10–40 min) [54]. However, inappropriate temperature or time for heating may cause color change or protein matrix breakdown, hence novel methods have been developed to perform this step, such as radiation and microwave [55, 56]. They all perform well for sterilization or second pasteurization (pasteurization is performed before fermentation). Meanwhile, the centrifugation in pre-treatment is another critical control point to remove the spores in the raw milk to avoid quality deterioration after packaging, just like traditional fermented milk production, where the parameters of the temperature and rotation speed is 50–65 °C and 4200–6300/min, respectively [54].

Despite having no live microorganisms in the fermented milk (including probiotics), the health effect of such a product may not be eliminated. Recently, the studies regarding postbiotics and parabiotics have been getting popular, and they are inactivated or killed probiotics [3], just like the microorganism in the pasteurized or sterilized fermented milk. Therefore, the health effect of postbiotics and parabiotics products cannot be determined, as not comparable with products containing live probiotics but needs more profound research to reveal the functional properties. The balance of nutritional values and the sale of the products need to be considered by manufacturers.

Another popular product, roasted-flavor fermented milk, has been developed based on Maillard reaction. This step was performed before homogenization, with the addition of glucose to promote the Maillard reaction to obtain the brown color and roasted flavor of raw milk [57]. Meanwhile, some concerns about this reaction are raised due to the production of harmful by-products, such as 5-hydroxymethylfurfural (HMF), glyoxal (GO), and methylglyoxal [58]. However, the harmful by-products all can be controlled after appropriate modification, such as keeping the product under 4 °C or setting a short shelf life [58]. Therefore, controlling such harmful by-products

must be considered when developing new products using new technology or additives.

5. There are many thickening methods in fermented milk production. Thickening is significantly important due to its wide application in thickened yoghurt production in both stirred and set types. The easiest way to improve or thicken the fermented milk texture is by adding thickener into the product at the beginning of the milk treatment. Usually, this kind of additive needs thorough mixing to have a better solution; hence homogenisation could be a good step to achieve this under such high pressure, and heat treatment can be done simultaneously. The rationale for thickener is that it could absorb or bind more water to enhance the protein matrix's strength and improve texture [59]. Therefore, appropriate agents that could absorb more water or strengthen the protein matrix are selected to improve texture, such as polysaccharides or proteins or both.

   In general, most thickeners do not influence the viability or the survival rate of probiotics. However, natural ingredients and naturally produced additives have been selected to replace artificial thickeners to improve the health value of the product, where their prebiotic potential has been revealed for these substances. Carob bean gum and chia seed mucilage have been proven to be able to enhance fermented milk's texture, but have no significant impact on probiotic growth [60, 61]. The bitter almond gum exudate and its conjugates with sodium caseinate (SBAG-SC) had performed preservative ability on the viability of *Lactobacillus acidophilus*, La-5, but possess lower prebiotic potential compared with inulin, as well as a comparable ability for preventing phase separation to fermented milk compared with carboxymethylcellulose (CMC) [62]. In fact, multi-types of polysaccharides have been adopted as encapsulated wall material for the protection of probiotics. This trend indicates that the dispersion of such polysaccharides, such as alginate, xanthan gum, gum arabic, and maltodextrin, could not only enhance the firmness of the fermented milk but also protect probiotics from the digestion of the human stomach to reach the intestinal tract efficiently, via the matrix and gel formed by them [63]. Probiotic encapsulation using polysaccharides could have texture modification ability as well. Low methoxyl pectin encapsulated *Bifidobacterium breve* could improve the viscosity of yogurt and the hardness when the capsule was applied before fermentation [64]. This result shows that polysaccharides' preservation and texture modification ability can co-exist, but their effect may vary when applied under different forms.

Meanwhile, a starch-pectin blend, in which the ingredients were both commonly used in fermented milk as texture modifiers, could form resistant starch and slow-digestible starch via their interaction (starch and pectin), possessing a synergistic effect on probiotics growth [65]. This capacity has broadened the horizon that the impact of thickeners may not act alone, but the interaction between various thickeners could benefit the growth or viability of probiotics. In addition, some prebiotics (inulin, tragacanth gum, gellan gum) could improve the rheological properties of fermented milk, such as firmness and apparent viscosity, and weaken the syneresis as biopolymers [66]. The application of prebiotics to improve the viability or count of probiotics should consider their texture enhancement effect for better performance, or avoid undesired quality deterioration.

Some intrinsic components of the milk, especially fortified milk protein, could enhance the texture of fermented milk through crosslink formation with polysaccharides by the starter culture. Polymerized whey protein could be a good thickener for set fermented goat milk due to its good performance with the adjunction of pectin (PWP) [67]. PWP has properties like low syneresis, desirable viscosity and hardness, but its retention ability for the population of probiotics has added value to this mixture [67]. PWP could retain the viable cell of *Lactobacillus acidophilus* above 10^6 cfu/mL for 4 weeks, which proves its effect on probiotic retention [67]. Milk protein concentration can be regarded as a protecting agent for encapsulating *Lacticaseibacillus paracasei*, combined with gellan-caseinate [68] (Kia et al., 2018). This shell material could reduce syneresis as well [68]. This phenomenon shows its capability to maintain the viability of probiotics during storage alongside the elevation of textural quality.

Recently, many physical technologies have been developed and applied to realize this target. They remove an appropriate amount of water or whey to thicken the milk without adding anything extra to provide a "thick" mouth sensation to consumers. These technologies include flash evaporation [69], freeze concentration [70], centrifugation concentration, and membrane filtration [71]. Their core mechanism is that they could improve the content of dry matter to provide a thick sensation to the consumers, whereas the higher dry matter content could benefit the viability of probiotics as well [32].

6. Sweetener is another vital ingredient in fermented milk due to the harsh sensation of lactic acid and other organic acids. It could reduce and balance such sensations by providing sweetness to consumers. Some sweeteners could also promote the growth of probiotics. Traditionally, sucrose (sugar) has been

used as a sweetener with the highest acceptance among other sweeteners, where the growth of probiotics can also be maintained and controlled. However, the recent trend of fermented milk requires healthier ingredients, where the calorie needs to be controlled. Hence, artificial sweeteners and natural sweeteners (low glycemic index) have been developed to replace sugar as sweeteners. Most of the commonly used artificial sweeteners, such as aspartame, neotame, sucralose, sorbitol, and polynols (xylitol, erythritol, maltitol and isomalt), have no influence on the growth of probiotics[72–77], but their health concern mainly resides at metabolism aspects. For natural sweeteners, honey is a popular natural sweetener that consumers welcome and accept widely when replacing sugar. It is shown that the addition of honey does not affect the viability of *Streptococcus* in starter culture and could improve the viability retention of *Bifidobacterium animalis* BB-12, which show the suitability of honey to be used as a healthier natural sweetener compared with sugar[78]. Stevia (steviol glycoside) is a leaf extract of Stevia rebaudiana, a popular natural and low-calorie sweetener. It has been proved that it could enhance gel matrix and probiotic growth (*lactobacillus acidophilus*) with no harm to the sensory properties of fermented milk [79]. Stevia could also maintain the survival rate of *lacticaseibacillus casei* above 9 logs CFU/ml for 28 days of storage. Fermentable fibre addition (red beetroot) could assist its prebiotic performance[80], which was mainly observed in *lacticaseibacillus casei*'s growth promotion[81]. However, some researchers reported that stevia has a bitter aftertaste[82], which makes its usage need further attention. Iso-maltulose, also known as palatinose, is a product of an enzymatic reaction (glucosyltransferases) from sucrose[83]. It has both sweetness and prebiotic potential, which could favour the growth of probiotics, including *lactobacillus acidophilus*, *lactococcus* sp. and *bifidobacterium animalis*, with preservation of their biofunctions[83, 84]. This characteristic has broadened the horizon of this sweetener and makes it possible to be regarded as a prebiotic sweetener. This multifunctional property could reduce the ingredients added to the fermented milk and favour the growth of probiotics for better performance.

As mentioned above, prebiotics is a kind of non-digestible component of the host by endogenous enzymes that benefits the modulation of intestinal microflora. Prebiotics usually appear as a carbohydrate that does not digest in the small intestine but is fermented in the colon. Sweetener is typically a kind of carbohydrate as well, which makes it possess the high prebiotic potential to benefit the growth of probiotics. Mogroside is an extract from *Siraitia grosvenorii* (monk fruit) with high sweetness intensity. It is claimed that it has no effect on

the fermented camel milk's organoleptic properties and could modulate the gut microbiota [85, 86]. Recent research revealed that the enzymatically modified mogroside combined with galactooligosaccharides (produced from mogroside and lactose combination by β-galactosidase) could improve the growth of gut microbiota, which includes *Bifidobacterium*, *Bacteroides*, *Enterococcus*, and *Clostridium coccoides* [87]. These examples indicate that using natural sweeteners has the advantages of low-calorie, prebiotic potential and high sweetness intensity, which makes them suitable to be used in many products and situations, especially probiotic fermented milk.

7. Many quality deteriorations may occur after product manufacturing and during storage. The deteriorations include texture, flavor, and color changes due to many factors. Here, we will briefly discuss some typical quality decline to give a comprehensive vision of quality control, but specified issue solution needs to be considered individually according to the situation. One of the most critical issues here is post-acidification. This phenomenon is mainly due to the growth of microorganisms during storage. Post-acidification can be affected by many factors, such as type of starter cultures, milk composition, temperature, and pH, homogenization and stirring, packaging material, and pre- and probiotics [88]. Here, the pre- and probiotic effects must be focused on due to their contradictory effect. Probiotics could metabolize some microbial inhibitory substances, such as bacteriocins or antimicrobial peptides, which could inhibit the growth of lactic acid producers. Hence, post-acidification can be assimilated. Further, the temperature set for probiotic growth may not favor the growth of lactic acid producers, where the pH dropping rate may be slowed under this condition, as mentioned above. However, prebiotics could accelerate the growth of many microorganisms, including lactic acid producers, as a promoting substance for microbial growth [88]. Even probiotics can produce more acid than usual [88]. Hence, controlling the production of such components is essential to ameliorate the post-acid effect. Other controlling methods mostly involve killing (or partially killing) the microorganisms in the fermented milk, but this does not meet some criteria of local regulations [88]. Hence, maybe the future direction could (1) genetically modify the microorganisms for reduced post-acidify capability [88] and (2) add live microorganisms (especially probiotics) back to the product after killing the intrinsic live cells and maintain the storage temperature at 4–5 °C for slowing the growth of such added microorganisms. However, the safety evaluation (such as antibiotic resistance, horizontal

gene transfer, etc.) would be more crucial to understanding the benefit of such products.

Other quality deterioration that can be discussed is based on the product. For example, set-fermented milk has issues with curd texture, whey isolation, undesired flavor, mold growth, and bad mouthfeel (sandy texture) [11]. For the curd texture and whey isolation, the main reason is the curd structure breakdown or inappropriate structure. The curd structure is affected by protein and polysaccharides cross-link. The protein content, protein quality (milk quality and composition), acid content, and microbial growth (polysaccharides production) may affect the structure. Among them, phage contamination may cause a significant quality issue by inhibiting the growth of microorganisms [11], which affect not only the curd but also other aspects, such as acid production, aroma component, etc. Phage is a type of virus with specificity to particular microorganism. It could lyse the microbial cell and kill them by leaching. Hence, the hygienic condition of the starter culture is important. Also, starter culture replacement and strain mixture are optional methods to avoid phage attacks due to their specificity [11]. The rest issues include undesired flavor, mold growth, and bad mouthfeel, possibly due to microbial contamination and raw milk quality deterioration (even mastitis milk has been used for manufacturing fermented milk products) [11]. Excessive hygienic practices and raw milk tests could prevent these issues.

Stirred fermented milk also has very similar issues to the set-counterpart. It is worth noting that stirring may introduce air into the product, which raises the possibility of whey isolation (due to air stratification) and microbial contamination (unclean air introduces yeast or mold into the product, especially the cross-contamination from other production lines which contain such contaminants) [11].

For probiotic beverages, the quality issues include live cell count, precipitation, fat isolation, flavoring ingredient quality, and microbial contamination [11]. Precipitation is a particular issue in probiotic beverages because observable precipitation in fermented milk is acceptable but not for beverages, hence appropriate methods to solve this issue are needed. Homogenization is an excellent way to mix ingredients and break protein (the main component of precipitate) to obtain a uniform beverage [11, 40]. This step is also used in Yakult® production [49]. Stabilizers and sugar are also available to facilitate homogenization [11, 40].

References

1. Marco ML, Sanders ME, Gänzle M et al (2021) The international scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on fermented foods. Nat Rev Gastroenterol Hepatol 18:196–208. https://doi.org/10.1038/s41575-020-00390-5
2. Sánchez B, de los Reyes-Gavilán CG, Margolles A, Gueimonde M (2009) Probiotic fermented milks: present and future. Int J Dairy Technol 62:472–483. https://doi.org/10.1111/j.1471-0307.2009.00528.x
3. Salminen S, Collado MC, Endo A et al (2021) The international scientific Association of Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of postbiotics. Nat Rev Gastroenterol Hepatol 18: 649–667. https://doi.org/10.1038/s41575-021-00440-6
4. Fisberg M, Machado R (2015) History of yogurt and current patterns of consumption. Nutr Rev 73:4–7. https://doi.org/10.1093/nutrit/nuv020
5. Ozen M, Dinleyici EC (2015) The history of probiotics: the untold story. Benefic Microbes 6:159–165. https://doi.org/10.3920/BM2014.0103
6. Tan SY, Dee MK (2009) Elie Metchnikoff (1845–1916): discoverer of phagocytosis. Singap Med J 50:456–457
7. Ebner S, Smug LN, Kneifel W et al (2014) Probiotics in dietary guidelines and clinical recommendations outside the European Union. WJG 20:16095. https://doi.org/10.3748/wjg.v20.i43.16095
8. Hill C, Guarner F, Reid G et al (2014) The international scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol 11:506–514. https://doi.org/10.1038/nrgastro.2014.66
9. CNFIA CNFIA (2021) Probiotic Foods (In Chinese) (T/CNFIA 131–2021)
10. Meng X, aili L, Jiao Y (2019) The processing technology of lactic acid bacteria food (in Chinese), 1st edn. Science Press, Beijing
11. Li X (2011) Technology of dairy products (in Chinese), 1st edn. Science Press, Beijing
12. Heck JML, van Valenberg HJF, Dijkstra J, van Hooijdonk ACM (2009) Seasonal variation in the Dutch bovine raw milk composition. J Dairy Sci 92:4745–4755. https://doi.org/10.3168/jds.2009-2146
13. Yoon JT, Lee JH, Kim CK et al (2004) Effects of Milk production, season, parity and lactation period on variations of Milk urea nitrogen concentration and Milk components of Holstein Dairy Cows. Asian Australas J Anim Sci 17:479–484. https://doi.org/10.5713/ajas.2004.479
14. Sakar U, Gupta AP, Sakar V et al (2006) Factors affecting test day milk yield and milk composition in dairy animals. J Dairying Foods HS 25:129–132
15. Filik G, Görgülü M, Boga M (2011) The changes of morning and afternoon milk composition of Holstein cows in different season. University of Osmangazi, Eskisehir
16. Bahashwan S (2014) Effect of cold and hot seasons on fat, protein and lactose of Dhofari cow's milk. Net J Agri Sci 2:47–49
17. Çobanoglu Ö, Gurcan EK, Çankaya S et al (2016) Effects of lactation month and season on test-day milkyield and milk components in Holstein cows. IJAR 51:952–955. https://doi.org/10.18805/ijar.11464
18. Hou J, Yang L (2010) The production technology of high quality raw milk (in Chinese), 1st edn. Chemical Industry Press, Beijing
19. van Vliet S, Provenza FD, Kronberg SL (2021) Health-promoting phytonutrients are higher in grass-fed meat and Milk. Front Sustain Food Syst 4:555426. https://doi.org/10.3389/fsufs.2020.555426
20. O'Callaghan TF, Hennessy D, McAuliffe S et al (2016) Effect of pasture versus indoor feeding systems on raw milk composition and quality over an entire lactation. J Dairy Sci 99: 9424–9440. https://doi.org/10.3168/jds.2016-10985
21. Yan H, Du W, Ji S et al (2022) Bacterial composition and interactions in raw Milk and teat skin of dairy cows. Fermentation 8: 235. https://doi.org/10.3390/fermentation8050235
22. Miller RA, Kent DJ, Boor KJ et al (2015) Different management practices are associated with mesophilic and thermophilic spore levels in bulk tank raw milk. J Dairy Sci 98:4338–4351. https://doi.org/10.3168/jds.2015-9406
23. Ross K (2021) Regulatory requirements for distribution of Milk for raw consumption in California
24. Liu H, Sun Y (2021) Comparative study on raw milk standards of China and other countries (in Chinese), 1st edn. China

Agricultural Science and Technology Press, Beijing

25. More SJ, Clegg TA, Lynch PJ, O'Grady L (2013) The effect of somatic cell count data adjustment and interpretation, as outlined in European Union legislation, on herd eligibility to supply raw milk for processing of dairy products. J Dairy Sci 96:3671–3681. https://doi.org/10.3168/jds.2012-6182
26. Kul E, Şahin A, Atasever S et al (2019) The effects of somatic cell count on milk yield and milk composition in Holstein cows. Vet Arhiv 89:143–154. https://doi.org/10.24099/vet.arhiv.0168
27. NHC (2010) National food safety standard: Fermented Milk (In Chinese)
28. Mohammadi R, Sohrabvandi S, Mohammad Mortazavian A (2012) The starter culture characteristics of probiotic microorganisms in fermented milks: characteristics of probiotic starters. Eng Life Sci 12:399–409. https://doi.org/10.1002/elsc.201100125
29. Tamime AY, Saarela M, Sndergaard AK et al (2006) Production and maintenance of viability of probiotic micro-organisms in dairy products. In: Tamime A (ed) Probiotic dairy products. Blackwell Science Ltd, Oxford, UK, pp 39–72
30. Shihata A, Shah NP (2002) Influence of addition of proteolytic strains of lactobacillus delbrueckii subsp. bulgaricus to commercial ABT starter cultures on texture of yoghurt, exopolysaccharide production and survival of bacteria. Int Dairy J 12:765–772. https://doi.org/10.1016/S0958-6946(02)00071-7
31. Mortazavian AM, Ghorbanipour S, Mohammadifar MA, Mohammadi M (2011) Biochemical properties and viable probiotic population of yogurt at different bacterial inoculation rates and incubation temperatures. Philipp Agric Scientist 92:111–116
32. Shafiee G, Mortazavian AM, Mohammadifar MA, et al Combined effects of dry matter content, incubation temperature and final pH of fermentation on biochemical and microbiological characteristics of probiotic fermented milk. Afr J Microbiol Res 10
33. Vinderola CG, Gueimonde M, Delgado T et al (2000) Characteristics of carbonated fermented milk and survival of probiotic bacteria. Int Dairy J 10:213–220. https://doi.org/10.1016/S0958-6946(00)00031-5
34. Vinderola CG, Costa GA, Regenhardt S, Reinheimer JA (2002) Influence of compounds associated with fermented dairy products on the growth of lactic acid starter and probiotic bacteria. Int Dairy J 12:579–589. https://doi.org/10.1016/S0958-6946(02)00046-8
35. Kailasapathy K (2006) Survival of free and encapsulated probiotic bacteria and their effect on the sensory properties of yoghurt. LWT Food Sci Technol 39:1221–1227. https://doi.org/10.1016/j.lwt.2005.07.013
36. Frakolaki G, Kekes T, Lympaki F et al (2022) Use of encapsulated *Bifidobacterium animalis* subsp *lactis* through extrusion or emulsification for the production of probiotic yogurt. J Food Process Engineering:45. https://doi.org/10.1111/jfpe.13792
37. Mohammadi R, Mortazavian AM (2011) Review article: technological aspects of prebiotics in probiotic fermented milks. Food Rev Intl 27:192–212. https://doi.org/10.1080/87559129.2010.535235
38. FAO/WHO (2003) Codex Alimentarius International Food Standards For Fermented Milks
39. Patrignani F, Burns P, Serrazanetti D et al (2009) Suitability of high pressure-homogenized milk for the production of probiotic fermented milk containing *lactobacillus paracasei* and *lactobacillus acidophilus*. J Dairy Res 76:74–82. https://doi.org/10.1017/S0022029908003828
40. Gu M (2009) Technical handbook for dairy engineers (in Chinese), 1st edn. China Light Industry Press, Beijing
41. Østlie HM, Treimo J, Narvhus JA (2005) Effect of temperature on growth and metabolism of probiotic bacteria in milk. Int Dairy J 15:989–997. https://doi.org/10.1016/j.idairyj.2004.08.015
42. Moslemi M, Mazaheri Nezhad Fard R, Hosseini SM et al (2016) Incorporation of Propionibacteria in fermented milks as a probiotic. Crit Rev Food Sci Nutr 56:1290–1312. https://doi.org/10.1080/10408398.2013.766584
43. Wardani SK, Cahyanto MN, Rahayu ES, Utami T (2017) The effect of inoculum size and incubation temperature on cell growth, acid production and curd formation during milk fermentation by Lactobacillus plantarum Dad 13. Int Food Res J 24:921–926
44. Luz C, Calpe J, Manuel Quiles J et al (2021) Probiotic characterization of lactobacillus strains isolated from breast milk and employment for the elaboration of a fermented milk product. J Funct Foods 84:104599. https://doi.org/10.1016/j.jff.2021.104599
45. Sun Y, Peng C, Wang J et al (2022) Mesopic fermentation contributes more to the formation of important flavor compounds and increased growth of lactobacillus casei Zhang than does high temperature during milk fermentation and storage. J Dairy Sci 105:4857–

4867. https://doi.org/10.3168/jds.2021-20949

46. Yoo S-H, Seong K-S, Yoon S-S (2013) Physicochemical properties of kefir manufactured by a two-step fermentation. Korean J Food Sci Anim Resour 33:744–751. https://doi.org/10.5851/kosfa.2013.33.6.744
47. Li F, Gong F (2021) Research and process development of fermented dairy products (in Chinese), 1st edn. Southwest Jiaotong University Press, Chengdu
48. Sadaghdar Y, Mortazavian AM, Ehsani MR (2012) Survival and activity of 5 probiotic lactobacilli strains in 2 types of flavored fermented milk. Food Sci Biotechnol 21:151–157. https://doi.org/10.1007/s10068-012-0019-z
49. Singapore Y (2022) Yakult product manufacturing process. Yakult Singapore, In. https://yakult.com.sg/manufacturing-process/. Accessed 25 Aug 2022
50. Wang J, Guo Z, Zhang Q et al (2010) Effect of probiotic *lactobacillus casei* Zhang on fermentation characteristics of set yogurt. Int J Dairy Technol 63:105–112. https://doi.org/10.1111/j.1471-0307.2009.00556.x
51. Zhang Y, Wang L, Zhang J et al (2014) Probiotic lactobacillus casei Zhang ameliorates high-fructose-induced impaired glucose tolerance in hyperinsulinemia rats. Eur J Nutr 53:221–232. https://doi.org/10.1007/s00394-013-0519-5
52. Bai M, Huang T, Guo S et al (2020) Probiotic lactobacillus casei Zhang improved the properties of stirred yogurt. Food Biosci 37:100718. https://doi.org/10.1016/j.fbio.2020.100718
53. Wang R, Li G, Wang P et al (2020) Effect of non-viable<i>lactobacillus Paracasei<i> L9 in Normal temperature yogurt on modulating gut microbiota and relieving constipation (in Chinese). J Food Sci Technol 38:95–101. https://doi.org/10.3969/j.issn.2095-6002.2020.03.012
54. Zheng W, Ba G, Zhao L (2014) The invention relates to a fermented dairy product which can be stored at room temperature and a coupled preparation method (in Chinese). 1–12
55. Yin X (2019) Study on compounding of lactic acid bacteria and long-term microwave sterilization Technology in set yak Yogurt under Highland anoxia environment (in Chinese). Master thesis. Lanzhou University of Technology
56. Luo D, Huang J, Deng K et al (2020) Irradiation sterilization technology applied to the normal temperature yogurt production (in Chinese). Food and Fermentation Sciences & Technology 56:41–47,59. https://doi.org/10.3969/j.issn.1674-506X.2020.05-007
57. Yang M, Zhao C, Shi C et al (2019) Optimization of Brown yogurt by design-response surface methodology (in Chinese). Food and Fermentation Sciences & Technology 55:21–28. https://doi.org/10.3969/j.issn.1674-506X.2019.03-005
58. Bi R, Lu J, Zhang S et al (2021) Changes of Maillard reaction by-products in brown yogurt under different storage conditions (In Chinese). Food Ferment Ind 47:116–124. https://doi.org/10.13995/j.cnki.11-1802/ts.024695
59. Cardines PHF, Baptista ATA, Gomes RG et al (2018) Moringa oleifera seed extracts as promising natural thickening agents for food industry: study of the thickening action in yogurt production. LWT 97:39–44. https://doi.org/10.1016/j.lwt.2018.06.028
60. Aydinol Sonmez P, Ozcan T (2021) Assessment of structure and sensory characteristics of reduced fat yoghurt manufactured with carob bean gum polysaccharides. Food Sci Technol 42:e61220. https://doi.org/10.1590/fst.61220
61. Atik DS, Demirci T, Öztürk Hİ et al (2020) Chia seed mucilage versus guar gum: effects on microstructural, textural, and antioxidative properties of set-type yoghurts. Braz Arch Biol Technol 63:e20190702. https://doi.org/10.1590/1678-4324-2020190702
62. Hashemi K, Hosseini E (2021) The stabilizing and prebiotic potential of water-soluble phase of bitter almond gum exudate in probiotic yogurt drink. Carbohydr Polym 9
63. Salama HH, Trif M, Rusu AV, Bhattacharya S (2022) Application of functional and edible coatings and films as promising strategies for developing dairy functional products—a review on yoghurt case. Coatings 12:838. https://doi.org/10.3390/coatings12060838
64. Li M, Jin Y, Wang Y et al (2019) Preparation of Bifidobacterium breve encapsulated in low methoxyl pectin beads and its effects on yogurt quality. J Dairy Sci 102:4832–4843. https://doi.org/10.3168/jds.2018-15597
65. Lin D, Ma Y, Qin W et al (2022) The structure, properties and potential probiotic properties of starch-pectin blend: a review. Food Hydrocoll 129:107644. https://doi.org/10.1016/j.foodhyd.2022.107644

66. Yu D, Kwon G, An J et al (2021) Influence of prebiotic biopolymers on physicochemical and sensory characteristics of yoghurt. Int Dairy J 115:104915. https://doi.org/10.1016/j.idairyj.2020.104915
67. Wang H, Wang C, Wang M, Guo M (2017) Chemical, physiochemical, and microstructural properties, and probiotic survivability of fermented goat milk using polymerized whey protein and starter culture Kefir Mild 01: FGM with PWP and Kefir Mild 01. J Food Sci 82:2650–2658. https://doi.org/10.1111/1750-3841.13935
68. Moghaddas Kia E, Ghasempour Z, Ghanbari S et al (2018) Development of probiotic yogurt by incorporation of milk protein concentrate (MPC) and microencapsulated *lactobacillus paracasei* in gellan-caseinate mixture. BFJ 120:1516–1528. https://doi.org/10.1108/BFJ-12-2017-0668
69. Kiesner C, Eggers R (1994) Concept of a sterile concentration process for milk by multistage flash evaporation. Chem Eng Technol 17:374–381. https://doi.org/10.1002/ceat.270170603
70. Sánchez J, Hernández E, Auleda JM, Raventós M (2011) Review: freeze concentration technology applied to dairy products. Food Sci Technol Int 17:5–13. https://doi.org/10.1177/1082013210382479
71. Meyer P, Hartinger M, Sigler S, Kulozik U (2017) Concentration of Milk and whey by membrane Technologies in Alternative Cascade Modes. Food Bioprocess Technol 10:674–686. https://doi.org/10.1007/s11947-016-1848-1
72. Başyiğit G, Kuleaşan H, Karahan AG (2006) Viability of human-derived probiotic lactobacilli in ice cream produced with sucrose and aspartame. J Ind Microbiol Biotechnol 33:796–800. https://doi.org/10.1007/s10295-006-0128-x
73. Esmerino EA, Cruz AG, Pereira EPR et al (2013) The influence of sweeteners in probiotic petit Suisse cheese in concentrations equivalent to that of sucrose. J Dairy Sci 96:5512–5521. https://doi.org/10.3168/jds.2013-6616
74. Costa GM, Paula MM, Barão CE et al (2019) Yoghurt added with lactobacillus casei and sweetened with natural sweeteners and/or prebiotics: implications on quality parameters and probiotic survival. Int Dairy J 97:139–148. https://doi.org/10.1016/j.idairyj.2019.05.007
75. dos Santos Filho AL, Freitas HV, Rodrigues S et al (2019) Production and stability of probiotic cocoa juice with sucralose as sugar substitute during refrigerated storage. LWT 99:371–378. https://doi.org/10.1016/j.lwt.2018.10.007
76. Kalicka D, Znamirowska A, Pawlos M et al (2019) Physical and sensory characteristics and probiotic survival in ice cream sweetened with various polyols. Int J Dairy Technol 1471–0307:12605. https://doi.org/10.1111/1471-0307.12605
77. Atallah AA, Ismail EA, Yehia HM et al (2022) Physicochemical, Microbiological and Microstructural Characteristics of Sucrose-Free Probiotic-Frozen Yogurt during Storage. Foods 11:1099
78. Varga L, Süle J, Nagy P (2014) Short communication: viability of culture organisms in honey-enriched acidophilus-bifidus-thermophilus (ABT)-type fermented camel milk. J Dairy Sci 97:6814–6818. https://doi.org/10.3168/jds.2014-8300
79. Ozdemir T, Ozcan T (2020) Effect of steviol glycosides as sugar substitute on the probiotic fermentation in milk gels enriched with red beetroot (Beta vulgaris L.) bioactive compounds. LWT 134:109851. https://doi.org/10.1016/j.lwt.2020.109851
80. Ozcan T, Ozdemir T, Avci HR (2021) Survival of *lactobacillus casei* and functional characteristics of reduced sugar red beetroot yoghurt with natural sugar substitutes. Int J Dairy Technol 74:148–160. https://doi.org/10.1111/1471-0307.12741
81. Sakr EAE, Massoud MI (2021) Impact of prebiotic potential of stevia sweeteners-sugar used as synbiotic preparation on antimicrobial, antibiofilm, and antioxidant activities. LWT 144:111260. https://doi.org/10.1016/j.lwt.2021.111260
82. Du X, Myracle AD (2017) Development and evaluation of kefir products made with aronia or elderberry juice: sensory and phytochemical characteristics. Int Food Res J 25:1373–1383
83. de Souza WFC, de Castro RJS, Sato HH (2022) Sequential optimization strategy for the immobilization of Erwinia sp. D12 cells and the production of isomaltulose with high stability and prebiotic potential. Bioprocess Biosyst Eng 45:999–1009. https://doi.org/10.1007/s00449-022-02719-7
84. Pranckutė R, Kaunietis A, Kuisienė N, Čitavičius D (2014) Development of Synbiotics with inulin, Palatinose, α-Cyclodextrin and probiotic bacteria. Pol J Microbiol 63:33–41. https://doi.org/10.33073/pjm-2014-005
85. Buchilina A, Aryana K (2021) Physicochemical and microbiological characteristics of camel milk yogurt as influenced by monk

fruit sweetener. J Dairy Sci 104:1484–1493. https://doi.org/10.3168/jds.2020-18842

86. Xiao R, Liao W, Luo G et al (2021) Modulation of gut microbiota composition and short-chain fatty acid synthesis by Mogroside V in an *in vitro* incubation system. ACS Omega 6:25486–25496. https://doi.org/10.1021/acsomega.1c03485
87. Muñoz-Labrador A, Lebrón-Aguilar R, Quintanilla-López JE et al (2022) Prebiotic potential of a new sweetener based on Galactooligosaccharides and modified Mogrosides. J Agric Food Chem 70:9048–9056. https://doi.org/10.1021/acs.jafc.2c01363
88. Deshwal GK, Tiwari S, Kumar A et al (2021) Review on factors affecting and control of post-acidification in yoghurt and related products. Trends Food Sci Technol 109:499–512. https://doi.org/10.1016/j.tifs.2021.01.057
89. NCCIH C (2019) Probiotics: what you need to know. National Center for Complementary and Integrative Health. https://www.nccih.nih.gov/health/probiotics-what-you-need-to-know. Accessed 25 Aug 2022
90. FSANZ F standards ANZ (2021) Supporting document 2, Microbiology risk assessment: L-lactic acid producing microorganisms, Safety & food technology Consultation paper, Proposal P1028—Review of the regulation of infant formula products
91. Canada H (2022) Probiotics monograph. Drug and Health Products. http://webprod.hc-sc.gc.ca/nhpid-bdipsn/atReq.do?atid=probio&lang=eng. Accessed 25 Aug 2022
92. NHC (2022) List of strains that can be used in food (in Chinese)
93. Sarkar S (2007) Potential of kefir as a dietetic beverage – a review. Br Food J 109:280–290. https://doi.org/10.1108/00070700710736534
94. Ali A-RA, Metwally AMM, Mahmoud AH, Attia HF (2011) Effect of feeding probiotics on rats' immunity and health conditions during pregnancy. FNS 02:96–104. https://doi.org/10.4236/fns.2011.22013
95. Tamime AY, Hickey M, Muir DD (2014) Strained fermented milks - a review of existing legislative provisions, survey of nutritional labelling of commercial products in selected markets and terminology of products in some selected countries. Int J Dairy Technol 67:305–333. https://doi.org/10.1111/1471-0307.12147
96. Arslan S (2015) A review: chemical, microbiological and nutritional characteristics of kefir. CyTA - Journal of Food 13:340–345. https://doi.org/10.1080/19476337.2014.981588
97. (1997) National Dairy Code, 1997 - Part II and III (amended July 2005). In: Goverment of Canada. https://agriculture.canada.ca/en/canadas-agriculture-sectors/animal-industry/canadian-dairy-information-centre/acts-regulations-codes-and-standards/national-dairy-code-part-ii-and-iii. Accessed 25 Aug 2022
98. FSANZ (2015) Food Standards Australia New Zealand Act:1991
99. FDA (2022) Federal Food, Drug, and Cosmetic Act
100. Mukherjee A, Gómez-Sala B, O'Connor EM et al (2022) Global regulatory frameworks for fermented foods: a review. Front Nutr 9:902642. https://doi.org/10.3389/fnut.2022.902642

Chapter 2

Probiotic Cheeses

Celso Fasura Balthazar, Julien Chamberland, and Marie-Claude Gentès

Abstract

Chemical and microbiological stresses experienced by probiotic bacteria in certain fermented milks, especially in low-pH products, led to loss of viability in commercial products in a strain- and product-dependent manner. In this scenario, cheeses appeared as a valid alternative for delivering probiotic bacteria. A large number of scientific reports showed the suitability of different kind of cheeses for protecting cell viability due to special characteristics of this food matrix, especially in fresh and semi-hard cheeses. Yet, certain technological characteristics of cheese manufacture should still be considered when designing a probiotic cheese such as salt content, heating of the curd, temperature during ripening, or cheese shelf life in order to improve the cell viability and the sensory features of the product. For the moment, the market success of probiotic cheese is still far behind that of probiotic fermented milks. The suitability of cheese for the inclusion of probiotics should be highlighted, leading to the development of novel functional cheeses. As well, there is no single recipe for selecting the appropriate probiotic for large-scale application. A deep overview of the process and factors to ensure viability of the probiotic throughout cheese processing and shelf life has to be performed, as well, consumer's acceptability, because probiotic selection should have minimal impact on taste and texture. Sensory testing and quality control plan are tools that the dairy industry can rely on to assess these changes and as a decision-making aid.

Key words Bacteria, Cheese, Dairy, Functional, Healthy, Probiotic

1 Introduction

Cheese production involves complex interactions between milk components, coagulant enzymes, and a wide diversity of microorganisms. Among them, lactic acid bacteria (LAB) in starter culture play a crucial role during all phases of cheese making and ripening processes. When LAB grow in milk, they convert lactose to lactic acid, acidifying the media and promoting curd syneresis through its demineralization. During ripening, LAB also influence the development of flavor, aroma, texture, and eye formation, depending on the starter selected and the cheese type produced. Moreover, health-promoting bacteria, so-called probiotic can be included in some starter cultures to provide additional functionality to the

Adriano Gomes da Cruz et al. (eds.), *Probiotic Foods and Beverages: Technologies and Protocols*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-3187-4_2,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2023

cheese. New cheese varieties, for example, those containing high levels of probiotic bacteria, have resulted from further development of starter cultures. While probiotic bacteria are best known in fermented milk and yoghurt, the interest in adding probiotics to cheese is increasing [1].

Probiotic is defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [2]. In order to provide a beneficial health effect, probiotics should be consumed daily and remain viable throughout the gastrointestinal tract. The recommended intake is around 8–9 $\log_{10}$ colony forming units per gram ($\log_{10}$ CFU/g^1). The health benefits of probiotic bacteria include: (a) improving intestinal tract health; (b) enhancing the immune system; (c) synthesizing and enhancing the bioavailability of nutrients; (d) reducing symptoms of lactose intolerance; (e) decreasing the prevalence of allergy in susceptible individuals; and (f) reducing the risk of certain cancers. In this sense, the most extensively studied and widely used probiotic bacteria are from genera *Bifidobacterium* and *Lactobacillus* [3].

Cheese as a delivery matrix for probiotic bacteria is based on the fact that cheese is universally accepted in many diets around the world [4]. It is worthy to note that the efficacy of probiotic intake on health is highly linked to the frequency of consumption. There is growing consciousness about the importance of the dose of probiotic bacteria, frequency, and duration of treatment required for different conditions in different population groups [1]. Then it is reasonable to choose those foods as a vehicle of probiotics that are regularly consumed. As well in technological terms, cheeses are good carriers to deliver probiotic bacteria to human due to its generally lower acidic content, higher solid content, lower O_2 concentration than traditional dairy probiotic carriers such as yogurt and fermented milk. The higher buffering capacity of cheese is also beneficial to protect the probiotic bacteria in the gastric juice. The challenge in producing probiotic cheese is survival of the probiotic organisms during the long shelf life of the product. Survival in a cheese matrix is strain dependent, and the selected probiotic bacteria should have a high acid- and salt-tolerance and be compatible with the cheese starter culture [5]. Furthermore, survival depends on the processing conditions, product matrix, and storage conditions. A low cooking temperature during cheese making, a high pH at the end of the curd acidification, a low oxygen and salt content in the cheese combined with low storage temperatures during maturation might be applied to maximize the survival rate of the probiotic bacteria [5].

An in vitro simulation of gastric digestion (3 h, pH 2, and 3) of Argentinean fresh cheese containing probiotic *Lactobacillus acidophilus*, *Lacticaseibacillus casei*, and *Bifidobacterium bifidum* [6] showed that the food matrix conferred a significant protection of cell viability compared to cells digested as pure cultures. Cell counts

were around 7 $\log_{10}$ CFU/mL for *Bifidobacteria* and around 4 $\log_{10}$ CFU/mL for *L. acidophilus* and *L. casei* at pH 2 whereas after exposure of 3 h at pH 3, counts of probiotic bacteria were above 7 $\log_{10}$ CFU/mL. Sharp et al. [7] compared the survival capacity of a strain of *L. casei* in yogurt and in low-fat Cheddar cheese submitted to gastric conditions at pH 2. The authors concluded that in terms of exposure to acidic conditions, Cheddar cheese could be a better dairy probiotic carrier, as *L. casei* viability decreased by around 2 $\log_{10}$ CFU/mL after 30 min and dropped by only 1 $\log_{10}$ CFU/mL after 2 h of exposure (final concentration of 4 $\log_{10}$ CFU/mL). In yogurt, viable counts diminished to less than 1 $\log_{10}$ CFU/mL just after 30 min of exposure [7]. Mäkeläinen et al. [8] published that the probiotic *Lacticaseibacillus rhamnosus* HN001 and *L. acidophilus* NCFM included in a semi-soft Gouda cheese survived in a model of the gastrointestinal tract. However, the cheese matrix did not appear to affect the probiotic survival because the probiotics in the cheese and in freezed-dried powders exhibited similar survival levels, although differences were observed in the magnitude of the stimulating effects on cell lines [9].

Thus, probiotic bacteria are normally added together with the starter culture. During cheese manufacturing, the concentration of milk components, namely casein and fat, allows for a lower inoculation rate if the selected strain and manufacturing parameters are optimal. The introduction of DVS cultures for direct inoculation of the cheese milk has allowed culture producers to launch new culture blends consisting of both thermophilic and mesophilic strains designed for special cheese types as well as special cultures for the production of probiotic cheese [5].

This chapter discusses about probiotic strain used to manufacture different cheese types and the survival of those probiotics, regarding actions taken to increase their viability. A section will also describe the limitations from research to industrial limitations as well as point out the main factors to consider for appropriate probiotic strain selection for industrial application with the aim to spread the development of probiotic cheese.

2 Material and Methods

2.1 Cheese Classification

Cheeses are made with two essential steps: (1) gelation of milk through enzymatic hydrolysis of κ-casein or acidification to pH 4.6 (isoelectric point of caseins); and (2) whey drainage of the resulting curd. However, differences in milk types and key cheese manufacturing parameters such as the use of different starter and/or adjuncts, fermentation conditions, renneting parameters, scalding temperature, salting method, ripening conditions, or even cheese shape contribute to explain the diversity in textures,

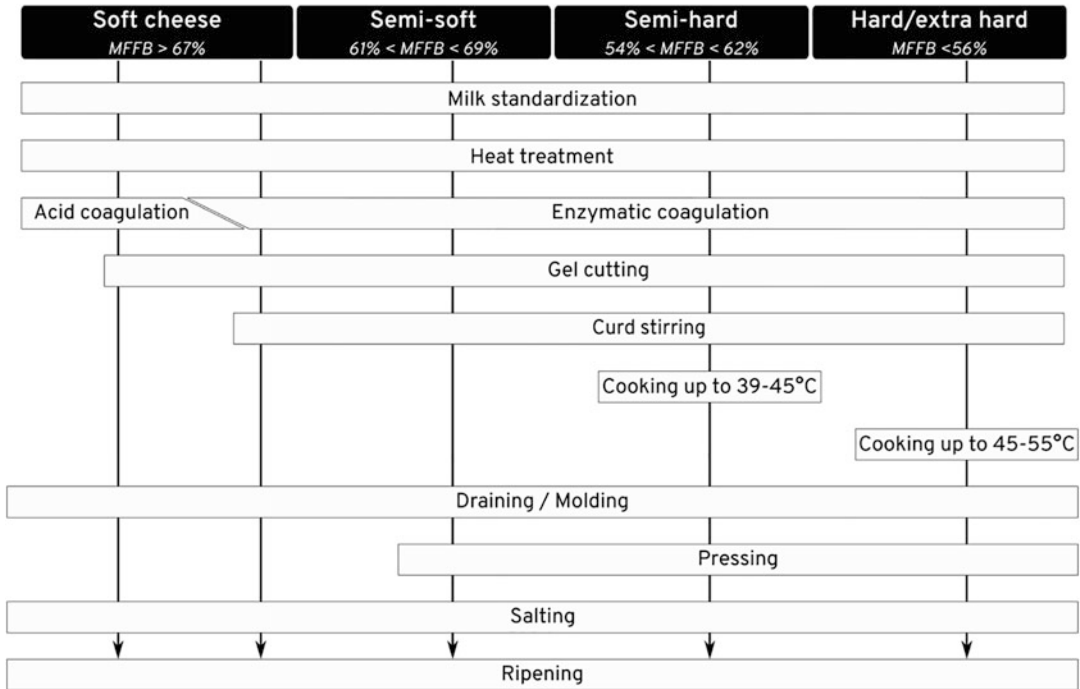

Fig. 1 Processing steps of major cheese types, based on their moisture content on a free fat basis (MFFB)

functional properties, flavors, and aroma of the many cheese types produced around the world [10]. These factors collectively influence final cheese composition and sensory characteristics.

Cheeses have been classified in relation to their chemical composition, the manufacturing processes, the presence of particular ripening microorganisms, maturity indices, etc. [11]. A classification based on the moisture content on a free fat basis (MFFB, Eq. 1) is particularly relevant when determining which cheese type should be manufactured to improve cell viability, because MFFB refers indirectly to the water activity.

$$\text{MFFB} = \frac{\%\text{Moisture}}{100 - \%\text{Fat}} \times 100 \qquad (1)$$

Cheeses can be classified as soft (MFFB >67%), semi-soft (MFFB between 61 and 69%), semi-hard (MFFB between 54 and 62%), hard (MFFB between and extra hard (MFFB content between 49 and 56%) and extra hard cheeses (MFFB <47%) (*see* Fig. 1) [12].

2.2 Cheese Manufacturing Process

As mentioned previously, in the initial stage of cheese production, a gel is formed from milk either by acid coagulation or by enzymatic coagulation (*see* Fig. 1). For the enzymatic coagulation, coagulating enzymes are used (e.g., chymosin) to hydrolyze the κ-casein fraction of casein micelles (breaking of the Phe_{105}-Met_{106} peptide

bond). The release of glycomacropeptide—a highly hydrophilyc fragment—destabilizes casein micelles, which results in the aggregation, and gelation of resulting casein aggregates. Once the gel formed is cut, many operating parameters (e.g., cutting size or firmness, duration of stirring steps, scalding time, and temperature) are selected to control the moisture loss of the resulting curd, depending on its moisture-content target. In fact, the cheese matrix, also known as paracaseinate matrix, is naturally prone to syneresis (whey expulsion). Consequently, from fresh to hard cheeses, the major difference between the manufacturing processes is that those for harder cheeses promote syneresis to reduce their moisture content. Syneresis is notably promoted by cutting a softer gel in smaller particles, by increasing scalding temperatures or by accelerating the acidification of the curd. The end of the draining step gives rise to the cheeses.

At this time, salting is carried out by means of brine or dry salting. Dry salting (salt being spread on the external surface of the product) is recommended for more humid cheeses, especially when promoting halotolerant microorganisms (e.g., *Penicillium camemberti*). Salting in saturated brine at >20% (w/w) NaCl is recommended for most cheeses, with the determination of the immersion period varying according to the type of cheese to be produced. Salted cheeses can be consumed fresh, or stored in controlled environment for cheese maturation that evidence the action of proteolytic or lipolytic enzymes from milk, coagulant, starters and ripening microorganisms commercial cultures, or introduced from the dairy environment, which provide the formation of unique flavor of each type of cheese [13].

Acid coagulation is generally achieved biologically through the production of lactic acid by LAB, but direct acidification with the addition of organic acids is possible. This coagulation type is used in a limited number of soft (fresh) cheese types (MFFB generally higher than 80%) such as cream cheese, quark (Tvorog), or labneh. The pH drops to around 4.6 (between 5 and 20 h), depending on the starter selected and the type of cheese produced. The following microorganisms are normally used: mesophiles *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis* biovar. *Diacetylactis*, and *Leuconostoc mesenteroides*; and thermophilic *Streptococcus thermophilus* and *L. delbrueckii* subsp. *bulgaricus* [13]. The acid gel (or lactic curd) cannot undergo intense draining steps as the enzymatic gel, because it is far less resistant to mechanical stresses due to its extensive decalcification. Lactic gels are concentrated by simple molding with ladles (or cheese draining bags) at the artisan scale, or by means of centrifugation or ultrafiltration at the industrial scale.

2.3 Incorporation of Probiotic Bacteria into Soft Cheese

With regard to the time of incorporation of probiotics into cheese during its manufacture, this generally occurs along with the addition of the starter cultures. In some cases, probiotics could be added after whey is drained from curd in order not to lose probiotic cells in whey [14]. For the following sections, manufacturing processes are presented for different cheese types.

2.3.1 Probiotic Cottage Cheese

Probiotic cottage cheese could be produced by addition of fermented cream at the end of the cheese manufacture and has the advantage to avoid cell loss in drained whey and the exposure of viable cells to high cooking temperatures. Some studies [15–17] used this strategy to add *Bifidobacteria* to cottage cheese. Cream (14% fat content with or without 1.8% salt) was inoculated with freeze-dried *B. infantis* ATCC 27920G and fermented at 37 °C until pH 4.5 was reached. The cultured dressing contained high levels of viable cells (ca. 8.5 $\log_{10}$ CFU/g), when salt was not present during fermentation but added once the desired pH was achieved. For cheese making, curd was formed at 30–32 °C from pasteurized skim milk inoculated with starter and supplemented with $CaCl_2$ solution (0.02% final concentration). Chymosin (100 μL/L milk) was added 1 to 1.5 h after starter addition and the curd was cut when pH reached 4.7. The curd was cooked at a rate to reach 50 °C to 55 °C in 1.5 h. Whey was drained and curd was washed with water at 21 °C and 5 °C. Curd was salted (0.6% w/w) and dressed with cream dressing fermented by *Bifidobacteria* to obtain approximately 4.5% fat (w/w) in the final cheese. The moisture of the cheeses produced ranged from 79.5 to 81.6%. However, the particular strain used did not adapt well to the food matrix since after 28 days of storage counts of viable cells were lower than 1 $\log_{10}$ CFU/g. However, after 14 days of storage at 4 °C, losses in cell viability around 2 to 3 log cycles were noted. Other types of soft cheeses can be elaborated using probiotic strains together with starter cultures or mixed with rennet [18].

2.3.2 Probiotic Minas Frescal

Minas is a typical Brazilian fresh unripened cheese with high moisture, low salt content, and absence of preservatives. These characteristics offer excellent conditions for survival and growth of probiotic strains [19]. The traditional procedure employed by Brazilian dairies for the manufacture of Minas fresh cheese implies the addition of mesophilic homofermentative lactic culture consisting of *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. Probiotic strains as *Lacticaseibacillus paracasei* subsp. *paracasei* LBC 82 could be added [20]. An alternative procedure employed by some Brazilian dairies is the direct acidification with lactic acid and without any addition of starter cultures. Pasteurized milk is inoculated with freeze-dried commercial cultures for direct vat inoculation. As soon as acidity reached about 20–22 °D, commercial rennet (3 g/L milk) and calcium chloride (0.2 g/L milk) were

added at 36 °C. After 40 min, the gel was cut gently into cubes (1 cm^2), allowed to drain, and placed in perforated circular molds. Cheeses were immersed in saturated brine for 30 min. After salting, cheeses were packaged in sealed plastic bags and stored at 5 °C for 21 days [19–21].

2.3.3 Probiotic Fresh Cheese

According to Roy et al. [22], fresh cheese was elaborated using ultrafiltered skim milk standardized at 30% solids and 20% fat, homogenized at 300 bar and pasteurized, followed by cooling milk up to 30 °C and starter and *Bifidobacteria* cultures were inoculated. Lyophilized cultures of *B. breve* R070 and *B. longum* R175 were used. Then milk was incubated at 30 °C for 10 h until pH reached a value of 4.6 and traditional fresh cheese procedure was performed. The product manufactured was kept at 4 or 12 °C for 57 days. The survival of *Bifidobacteria* at 4 or 12 °C was above 6 log_{10} CFU/g until day 15 of storage and gradually lost viability. After 22 days, probiotic bacteria counts were below the claim to be considered probiotic, and by day 50 of storage, *Bifidobacteria* were no longer detected. In another study with Argentinean fresh cheese containing *L. casei*, *L. acidophilus*, and *Bifidobacteria*, the cells' quantification decreased after 60 days of storage at 5 °C to less than 1.2 log_{10} CFU/g [6].

2.3.4 Probiotic Crescenza

Crescenza is a soft, rindless, Italian cheese (MFFB ~88%) with a short ripening time. Whole bovine milk was pasteurized and cooled to 35 °C. A direct-to-vat, freeze-dried *Streptococcus thermophilus* culture was added along with *B. bifidum*, *B. infantis*, and *B. longum*, initial concentrations of 6 and 7.5 log CFU/mL, respectively for starter and probiotics. Liquid calf rennet (3 mL, 20% pepsin, 80% chymosin) was immediately added and curd was formed in approximately 25 min to be cut to a size of 1.5 to 2.0 cm. After a 60-min holding period, the curd was then cut to a final size of ca. 0.5 to 1.0 cm and warmed at 35 °C for 150 min. The cheese was salted by immersion in 16 to 18% (w/w) NaCl brine for 1 h at 15 °C. The cheese was ripened for 10 days at 5 °C and subsequently stored at 6 °C for 4 days, which corresponded to commercial storage for this type of cheese. At day 14, the moisture content ranged from 61.7 to 63.0% and initial counts of *Bifidobacteria* were above 7 log CFU/g as in the production day [23].

2.3.5 Probiotic Turkish Cheese

Turkish Beyaz (MFFB ~73%), Kasar (MFFB ~74%) and Tulum (MFFB ~73%) cheeses are also considered good matrices for probiotic bacteria delivery. Studies indicated that $CaCl_2$ addition to pasteurized milk inoculated with *Limosilactobacillus fermentum* (AB5–18 and AK4–120) and *Lactiplantibacillus plantarum* (AB16–65 and AC18–82) probiotic strains with commercial starter mix consisting of *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* (1% according to the manufacturer) were incubated until the pH

reached ca. 5.9–6.3, then chymosin (20 mL) was added to milk to allow coagulation for 60 min. After that, curd was cut and rested in the whey for 5–10 min, followed by drainage without pressure for 30 min, and pressed (40 kg weights for 100 L milk) for 180 min. When the curd reached the appropriate strength, the cheese cloths were opened and the cheeses were cut into cubes. The cubes were brine-salted. Then, cheeses were kept at 37 °C for 12–18 h and the brined cheese blocks were packed in plastic bags containing brine and sealed with heat. Cheeses were ripened at 4 °C for 120 days, when moisture content ranged from 58.6 to 68.2%. Counts of total *Lactobacilli* remained up to 8 log_{10} CFU/g [24] (*see* **Note 1**).

2.4 Incorporation of Probiotic Bacteria into Semi-Soft Cheese

Festivo cheese is a semi-soft Finnish cheese with high moisture content (~62% w/w), in which pasteurized milk is inoculated at 32 °C with starter cultures containing *Lactococci*, *L. acidophilus*, and *Bifidobacterium* sp.. After 30 min of coagulation action, the curd is cut and part of the whey removed, followed by temperature raise up to 35 °C and curd stir. The whey is drainage and the cheese is pressed for 3 h and further rests in brine overnight. During this procedure the pH drops around one unit (from 6.45 to 5.20) and cheese is wrapped in plastic for storage about 10 °C for 4 months. *L. acidophilus* and *Bifidobacterium* spp. probiotic strains lost viability by ca. half log cycle compared to the initial levels of inoculation during cheese manufacture [25].

White cheese is a common semi-soft cheese manufactured in dairy industries, in which some studies [25–28] mixed probiotic strains with mesophilic starter and chymosin (amount varies according to manufacturers). Coagulation of milk takes place in 60 min and coagulum is cut with a knife into small cubes for curd to be allowed to stand in the whey for 5 to 10 min. Then, the curd is transferred to mold at pH 6.4 and the surfaces of the cheese is covered with cheesecloth, drained without pressure for 20 min, and pressed for 2.5 h. The cheese mass is cut into cubes (7 × 7 × 7 cm) for brine-salting (13% [w/w] NaCl) for 13 h. In sequence the cheese is kept at room temperature for 6 h to rest and, finally being ripened at 4 °C for 90 days. The probiotic inoculum should achieve initial counts of log 9 CFU/g, because during brining and storage at 4 °C, probiotic cells lose viability approximately by 2 to 3 log_{10}. Thus that procedure assures probiotic levels until the end of shelf life. The authors found that the decrease in the colony counts of *Bifidobacterium* and *L. acidophilus*, La-5, was faster during the first 30 days of storage [25–28].

Pategrás cheese is an Argentinean semi-soft cheese produced with pasteurized cow's milk, which is cooled to 37 °C for calcium chloride and lyophilized starter (*S. thermophilus*) addition. In this moment of manufacture, probiotic cultures (*L. acidophilus, L. paracasei* and *B. lactis*) are added and after 15 min, there is addition of chymosin for proper formation of curd in appropriate

strength. The curd is cut and stirred in whey under heat at the rate of 1 °C/min until 45 °C for 15–20 min to reduce the moisture content in the final product. Then, the curd is separated from whey, molded and pressed during 24 h. Young cheeses are salted in saturated brine 20% (w/w), pH 5.40, for 24 h and ripened for 2 months at 12 °C. Both probiotic strains are able to grow approximately 1.5 log cycles during cheese manufacture and counts remain above 8 $\log_{10}$ during the 60 days of refrigerated storage [29–31].

Feta cheese is a semi-soft, white cheese, usually ripened in brine, which is originally produced in Greece using ewes' or goats' milk, or both, by coagulation using only rennet [32]. Probiotic Feta cheese could be obtained by pasteurized ewes' milk added with *L. casei* ATCC 393 and rennet. The inoculated milk is left undisturbed for 2 h for curd formation. The curd is cut and cloth-filtered overnight at room temperature (18 to 22 °C). The effect of salt addition on cheese quality characteristics was studied by rubbing 10 g of salt per 100 g of cheese on the surface. Ripening of the cheeses was monitored at 4 to 6 °C for 71 days. The probiotic strain used showed a satisfactory survival in Feta cheese either in the products with or without salt added: above log 6 CFU/g [32].

A mix of *L. acidophilus, B. longum*, and *B. lactis* were incorporated directly into lamb rennet paste as an approach to not modify the traditional step in pasta filata cheese production [33]. Pasta filata cheese is produced by thermiziation and texturization process, cooking curd in hot water (or whey) by mechanically mixing to achieve plastic consistency and extruded into different shapes and size. The method resulted in higher structural uniformity, lower friability, and higher creaminess and graininess cheese, presenting high levels of probiotic cells, in particular *Bifidobacteria*, throughout 2 weeks of ripening. Furthermore, the peptide profile of the pasta filata ewe ripened cheese highlighted specific peptides derived from the presence and activity of probiotic bacteria. In particular, the presence of bioactive sequences in long-ripened cheeses demonstrated the ability of probiotics to control and enhance the proteolytic process and generate peptides in the cheese matrix that could be delivered upon cheese consumption [33].

As described above, many studies reported successful production of probiotic semi-soft cheeses in adequate amount of viable probiotic cells in the end of storage, despite losses during salting and/or ripening (*see* **Note 2**).

2.5 Incorporation of Probiotic Bacteria into Semi-Hard and Hard Cheeses

For semi-hard and hard cheeses, probiotic strains are added with starter during cheese manufacture. Some time is usually required for strains to be activated and grown and acidify the media at temperatures around 30 °C, depending on the type of cheese. Then the procedure follows each type of cheese production established standards (*see* **Note 3**).

During cheddar cheese manufacture, mesophilic starter LAB (*L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*) are added to pasteurized cow's milk followed by rennetting. For probiotic cheddar cheese production, probiotic strains are added together with starter cultures in general. The mixture is allowed to set and form curds at a temperature of around 29–31 °C for 30 to 40 min. The curd is cut and allowed to stand for approximately 15 min. Then, the curd is cooked around 39 °C for 20–60 min; when the whey acidity is about pH 6.1–6.4 at the end of cooking phase, it has to be drained. Cheddaring is a unique step in cheddar cheese making to give the typical cheddar flavor. Thus, loaves of curds are cut about 15 cm wide along each side of the vat. After 10 min, the loaves are turned and stacked every 10 min. This process is complete when the pH is around 5.3–5.4. A curd mill is used to cut the loaves into cubes during constant stirring and salt is added up to 1 to 3% (w/w). Following, the curds are placed into molds and pressed to form blocks of cheddar cheese. Then, ripening takes place for different periods depending on the extent of ripening desired. The majority of the reports suggest that Cheddar cheese was successful in relation to the maintenance of cell viability for relatively long periods such as 9 or 15 months. An appropriate selection of starter/probiotic culture combination and adequate control of cheese and storage variables are a prerequisite for the successful incorporation of probiotics into cheese [34, 35] (*see* **Notes 4** and **5**).

To produce a probiotic caprine semi-hard cheese, goat milk is pasteurized and cooled to 32 °C for $CaCl_2$, a mixed-mesophilic starter culture, *B. lactis* and *L. acidophilus* addition. Double-strength calf rennet is added and curd formed in a min. The curd is cut and temperature is raised slowly to 38 °C within 20 min. The first whey drain occurs and the resulting curd is maintained at 38 °C for additional 20 min. After the second whey drainage, the curd is placed in hoops and held at 40 °C throughout pressing and held for 3 h) for then being brined. Cheese is ripened for 70 days at 6 °C. The resulting salt and moisture contents are about 3.5% and 37.6 to 43.2%, respectively, and cheese storage is until refrigeration. A significant loss in cell viability of 1.5 and 1 $\log_{10}$ CFU/g was observed for *B. lactis* and *L. acidophilus* after storage [36].

Canestrato Pugliese cheese is an Italian semi-hard cheese, for which sheep milk is used. The probiotic version was elaborated adding *B. bifidum* Bb02 and *B. longum* Bb46 to starter *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. The traditional protocol was modified in order to improve probiotic cell viability. Instead of heating the curd in hot whey at 80 °C for 30 s, the probiotic curd was kept to 50 °C for 2 min, and then held at 40 °C for 5 h to limit acidification by the starters. The salting was carried out by spreading salt over the surface and cheeses were ripened at 12 °C for 3 months. By month 2 of storage, *Bifidobacteria* counts decreased by 1.7 to 2.8 $\log_{10}$ cycles, indicating rather poor viability of these strains in this particular product.

Etchepare et al. [37] and Mirzaei et al. [38] have used resistant starch to microcapsulate probiotic bacteria (*L. acidophilus*, and

B. bifidum, *L. casei*, and/or *B. lactis*, respectively) in cheeses, which helped avoid viability losses during long-period storage and provided thermal stability improvement and protection against simulated gastrointestinal stress. Also the encapsulation of probiotics into rennet paste was investigated on Pecorino cheese manufactured from Gentile di Puglia ewe milk at different maturation times. The probiotic-containing alginate beads underwent a progressive disaggregation process, leading to the liberation of live microorganisms. In this study, after 60 days of ripening, the beads disappeared, and the probiotic cells remained at a level of about 7 $\log_{10}$ CFU/g of cheese up to 120 days of ripening [39]. Therefore, encapsulation may sustain the production of long-ripened cheese, maintaining high levels of live cells throughout the maturing process [40].

Probiotic strains in cheese reach levels above 6 $\log_{10}$ CFU/g, not modifying gross composition, nitrogen fractions, lipolysis, fatty acid profiles including conjugated linoleic acids (CLA) and volatile profile. The sensorial profile of the probiotic ovine cheese after 45 days of ripening was also influenced, showing lower humidity and gumminess and higher ratings for salty and pungent attributes compared to the control cheese [41]. The ability of *L. acidophilus* to enrich the cheese matrix with a great amount of free fatty acids (FFA) and CLA was further confirmed in a study of probiotic Pecorino foggiano cheese [42, 43]. *Bifidobacteria* added in cheese was able to produce high levels of linoleic acid as well as to induce greater proteolysis that was associated with lower cheese hardness. A study highlighted that probiotic bacteria may not survive in high numbers when freely incorporated into dairy products, and encapsulation was proposed as a method to increase the survival and delivery of probiotics [44, 45].

2.6 From Research Laboratory to Large-Scale Production

Despite the numerous literature on probiotic cheese, only a few are available regarding the key elements to consider to scale-up from laboratory research to large-scale production. Commercial probiotic cheeses are already sold in market around the world proven the feasibility.

This practical guide will then focus on the various points to consider before developing probiotic cheeses. Nevertheless, there is no one-size-fits-all probiotic cheese recipe. Selection of the appropriate strain must take into account cheese processing, biocompatibility of probiotic strain with other cultures, desired sensory properties and use once in the consumer's hand.

At laboratory scale, it is possible to control and easily modify the environment which is much more challenging under industrial reality. Minimal change on process increases the success of viable probiotic application. As previously stated, different cheese types have been tested for probiotic survival (Table 1). To select the appropriate probiotics for the right application, the first step is to review the cheese process and point out the possible steps that

Table 1
Probiotic strains used in different types of cheese

	Cheese type	Probiotic	Viability/Storage	Reference
Soft cheeses	Mina Frescal cheese	*Lacticaseibacillus* casei 01	8 log_{10} CFU/g / 28 days	Sperry et al. [19]
	Fresh cheese	*Bifidobacterium breve* R070 *B. longum* R175	6 log_{10} CFU/g / 15 days	Roy et al. [22]
	Beyaz, Kasar, and Tulum cheeses	*Limosilactobacillus fermentum* (AB5–18 and AK4–120) *Lactiplantibacillus plantarum* (AB16–65 and AC18–82)	8 log_{10} CFU/g / 120 days	Kılıç et al. [24]
	Boursin cheese	*B. animalis* subsp. lactis BB-12 *Lacticaseibacillus rhamnosus* LRB 10 C109721A	>7log_{10} CFU/g/ 35 days	Martins et al. [56]
	Soft goat cheese	*L. plantarum* 564	8.82 log_{10} CFU/g/ 42 days	Radulović et al. [57]
Semi-soft cheeses	Petegrás cheese	*L. paracasei* subsp. *paracasei* *B. lactis* BB-12	8 log_{10} CFU/g / 60 days	Bergamini et al. [31]
	Argentineanm cheese	*Lactobacillus acidophilus* LA-5 *B. lactis* BB-12	6 log_{10} CFU/g / 15 days	Perotti et al. [58]
	Feta cheese	*Propionibacterium freudenreichii* subsp. *shermanii* LMG 16424	9 log_{10} CFU/g / 60 days	Angelopoulou et al. [59]
	Coalho goat cheese	*L. mucosae* CNPC007	>8 log_{10} CFU/g / 28 days	Moraes et al. [60]
	Scamorza cheese	*B. longum* 46, *B. lactis* BB-12, *L. acidophilus* LA-5	*L. acidophilus*: 7.5 log_{10} CFU/g / 15 days *B. longum* and *B. lactis*: 9.9 log_{10} CFU/g /15 days	Albenzio et al. [33]
Semi-hard cheeses	Cheese	*L. rhamnosus* EM1107		Rodrigues et al. [61]
	Low-fat cheddar cheese	*L. casei* 334e	7 log_{10} CFU/g/ 90 days	Sharp et al. [7]
	Low-fat cheddar cheese	*B. animalis* subsp. *lactis* *L. rhamnosus* *L. paracasei/* *L. plantarum*	>4 log_{10} CFU/g/ 120 days	Demers-Mathieu et al. [54]
	Cheedar cheese	*L. rhamnosus* DPC7102	>7 log_{10} CFU/g/ 180 days	Leeuwendaal et al. [55]
Hard cheeses	Ovine cheese	*L. acidophilus LA-5, B. longum* and *B. lactis* BB-12	7 log_{10} CFU/g/ 45 days	Santillo & Albenzio [41]
	Pecorino cheese	*L. acidophilus*, *B. longum*, and *B. lactis no strain number?*	*L. acidophilus*: 8 log_{10} CFU/g / 60 days *B. longum* and *B. lactis*: 9 log_{10} CFU/g/60 days	Santillo & Albenzio [42] Santillo et al. [43]

(continued)

Table 1 (continued)

Cheese type	Probiotic	Viability/Storage	Reference
Pecorino cheese	*L. acidophilus*, *B. longum*, and *B. lactis no strain number?*	*L. acidophilus*: 8 $\log_{10}$ CFU/g/ 30 days *B. longum* and B. lactis: 9 $\log_{10}$ CFU/g/30 days	Albenzio et al. [62]
Pecorino cheese	*L. acidophilus*, *B. longum*, and *B. lactis no strain number?*	*L. acidophilus*: 7.52 $\log_{10}$ CFU/g/ 120 days *B. longum* and *B. lactis*: 6.84 $\log_{10}$ CFUg/ 120 days	Santillo et al. [39]
Canestrato Pugliesa cheese	*B. bifidum* Bb02 *B. longum* Bb46	> 6 $\log_{10}$ CFUg/ 56 days	Corbo et al. [63]

might affect the probiotic viability such as cooking, salting, storage time, and temperature [46]. Probiotic has to be heat-, acid- or salt-tolerant depending on the process. For instance, heat-resistant probiotics might be more adapted for pasta filata which involved a stretching curd step at high temperature (>80 °C) [47]. In addition, the cheese might be used at the consumer's hand for grating dish or pizza topping where high temperatures are used. *Bacillus coagulans*, a known heat-resistant probiotic strains, have been successfully added during curd fusion (>90 °C) of processed cheese [48].

The final pH and salt level of the cheese are also important to take into consideration [5]. Salt-resistant probiotic strain has to be selected for brined-cheese [28]. As the pH of acid curd is lower than 4.6, an acid-resistant probiotic strain should be selected. Another important element to consider is the biocompatibility of probiotics with the starter or the other adjunct cultures. Microorganisms can release metabolites that impart the growth of others. Though, an in vitro test can be performed to assess the different combinations of cultures/probiotics prior to large-scale test [49]. The method consists of simulating a Cheddar cheese fermentation process with the first culture (starter). A second fermentation is carried out with the other cultures (or probiotics) in the fermented whey obtained during the first fermentation. Growth kinetic is monitored by spectrophotometry. The biocompatibility of starter (*Lactococci*) and various commercial probiotics (*Lacticaseibacillus rhamnosus* GR-1, *L. rhamnosus* GG, *L. rhamnosus* R0011, *Lactobacillus helveticus* R0052, *L. acidophilus* LA-5 and *B. animalis* ssp.

lactis BB-12) was successfully evaluated by this method as selection tool prior to Cheddar cheesemaking at pilot plant scale [50].

From a technical point of view, the expertise of the quality control staff to monitor probiotic population throughout the shelf life as well as the required material to perform enumeration analysis has to be in place. Additional cost for training and equipment (e.g., autoclave to sterilize the culture media) has to be taken in consideration when developing probiotic cheese (*see* **Note 6**).

From consumer's point of view, the most important criterion for acceptability on functional food is taste [51]. Even though there is an added benefit to cheese, it has to taste good or even better than the traditional version. It is important to select probiotic that will not modify cheese sensory properties. For instance, *Bifidobacteria* sp. produces acetic acid that could impart an unpleasant or unusual flavor in cheese [52]. Sensorial evaluation testing is then critical. Performing laboratory-scale sensory testing is not straightforward due to ethical considerations. Research approval from ethical committee is required to conduct professional sensory testing [53]. At the opposite, the dairy plant has quality control plan in place to produce safe cheese according to strict regulations. Another key element is regarding assessing the functionality once in consumer's hand. In other words, it is expected that a low-moisture mozzarella will be used on pizza topping. The viability of the probiotic has also to be evaluated under these conditions to ensure the required minimal concentrations after this cooking step.

To summarize, there is no single recipe for selecting the appropriate probiotic for large-scale application. A deep overview of the process and factors to ensure viability of the probiotic throughout cheese processing and shelf life has to be performed. Consumer's acceptability is also key. Probiotic selection should have minimal impact on taste and texture. Sensory testing and quality control plan are tools that the dairy industry can rely on to assess these changes and as a decision-making aid (*see* **Note 7**).

3 Notes

1. Fresh cheeses are good matrix to deliver probiotics due to their high-water activity, pH below 5.0 (4.5–4.6), low salt content, absence of preservatives, and because they are not ripened. These cheeses are thus stored at refrigeration temperatures (<4 °C) and are consumed rapidly.
2. In regard to semi-soft cheeses, emulsion and extrusion microencapsulation techniques might be an effective way to improve probiotic cells' viability and to minimize losses during salting and/or ripening period.
3. The manufacture of probiotic semi-hard or hard cheeses must consider the selection of the most robust probiotic strains that are capable of surviving the salting step and long ripening

periods. It is recommended to reduce the salt content of the cheese, or to use encapsulated probiotics.

4. Sharp et al. [7], Demers-Mathieu et al. [54] and Leeuwendaal et al. [55] indicated Cheddar cheese as a good food matrix to deliver probiotic bacteria to the host. Probiotic bacteria showed good viability after 90 (7 log_{10} CFU/g), 120 ($>$ 7 log_{10} CFU/g), and 180 ($>$ 4 log_{10} CFU/g) days of ripening, respectively.
5. Sharp et al. [7] and Leeuwendaal et al. [55] also presented Cheddar cheese as protective matrix for probiotic delivery to host, being those viable at 4 log_{10} and 6 log_{10} CFU/g after simulated digestion, respectively.
6. The obstacle to manufacture probiotic cheeses, especially for semi-hard and hard cheeses, is to keep the adequate number of viable probiotic bacteria in cheese to be claimed as probiotic food due to be more challenging for probiotic bacteria survival in low moisture environment such as hard cheeses. Thus, probiotic concentrations in these cheeses have to be raised in the future in order to assure minimal daily intake of probiotics for nutritional health.
7. Since it is not easy to modify or to introduce new and persistent dietary habits in consumers, the success of the incorporation of probiotic bacteria into cheeses should be done by informing consumers about the advantages of these new products. The practical guide aimed to shed light on the key elements to consider for large-scale development from technical point of view to consumer acceptability in order to help widespread commercial probiotic cheese application.

References

1. Cruz AG, Ranadheera CS, Nazzaro F, Mortazavian A (2021) Probiotics and prebiotics in foods challenges, innovations, and advances. Academic Press, Elsevier Inc., London, UK
2. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Salminen S (2014) The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Na Rev Gastroenterol Hepatol 11(8): 506–514
3. Balthazar CF, Guimarães JF, Coutinho NM, Pimentel TC, Ranadheera CS, Santillo A, Albenzio M, Cruz AG, Sant'Ana AS (2022) The future of functional food: emerging technologies application on prebiotics, probiotics and postbiotics. Compr Rev Food Sci Food Saf 21:2560–2586. https://doi.org/10.1111/1541-4337.12962
4. Planzer SB, Gomes da Cruz A, Santana AS, Silva R, Moura MR, de Carvalho LM (2009) Food safety knowledge of cheese consumers. J Food Sci 74:28–30
5. Høier E, Janzen T, Rattray F, Sørensen K, Børsting MW, Brockmann E, Johansen E The production, application and action of lactic cheese starter cultures. In: Law BA, Tamime AY (eds) Technology of cheese making, 2nd edn. Blackwell Publishing Ltd: UK. ch 5, pp 166–192
6. Vinderola G, Prosello W, Ghiberto D, Reinheimer J (2000) Viability of probiotic (Bifidobacterium, Lactobacillus acidophilus and Lactobacillus casei) and nonprobiotic microflora in Argentinian Fresco cheese. J Dairy Sci 83:1905–1911
7. Sharp MD, McMahon DJ, Broadbent JR (2008) Comparative evaluation of yogurt and

low-fat Cheddar cheese as delivery media for probiotic *Lactobacillus casei*. J Food Sci 73: 375–377

8. Mäkeläinen H, Forssten S, Olli K, Granlund L, Rautonen N, Ouwehand AC (2009) Probiotic lactobacilli in a semi-soft cheese survive in the simulated human gastrointestinal tract. Int Dairy J 11:675–683
9. Vinderola G, Burns P, Reinheimer J (2010) Technology and stability of probiotics in probiotic and prebiotic cheeses. In: Shah NP, Cruz AG (eds) Probiotic and Prebiotic Foods. Nova Science Publishers, Inc.: USA. ch 8,, pp 260–295
10. Heller KJ, Bockelmann W, Schrezenmeir J, de Vrese M (2008) Cheese and its potential as a probiotic food. In: Farnworth ER (ed) Handbook of fermented functional foods. CRC Press, Boca Raton, USA, pp 243–266
11. McSweeney PLH, Ottogalli G, Fox PF (2017) Diversity and classification of cheese varieties: an overview. In: McSweeney PLH, Fox PF, Cotter PD, Everett DW (eds) Cheese, 4th edn. Academic Press. Ch 31
12. Pecorelli I, Branciari R, Roila R, Ranucci D, Bibi R, Asselt M, Valiani A (2020) Evaluation of Aflatoxin M1 enrichment factor in different cow milk cheese hardness category. Italian J Food Safety 9:8419
13. Johnson M, Law BA (2010) The origins, development and basic operations of Cheesemaking technology. In: Law BA, Tamime AY (eds) Technology of cheese making, 2nd edn. Blackwell Publishing Ltd: UK. ch 2,, pp 68–97
14. FDA (2022) Cheeses and related cheese products. In: CFR - Code of Federal Regulations Title 21. Food and Drugs Chapter I. Food and Drug Administration Department of Health and Human Services subchapter B - Food for Human Consumption. Available https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=133
15. Blanchette L, Roy D, Gauthier SF (1995) Production of cultured cottage cheese dressing by bifidobacteria. J Dairy Sci 78:1421–1429
16. Blanchette L, Roy D, Bélanger G, Gauthier SF (1996) Production of cottage cheese using dressing fermented by bifidobacteria. J Dairy Sci 79:8–15
17. O'Riordan K, Fitzgerald GF (1998) Evaluation of bifidobacteria for the production of antimicrobial compounds and assessment of performance in cottage cheese at refrigeration temperature. J Appl Microbiol 85:103–114
18. Masuda T, Yamanari R, Itoh T (2005) The trial for production of fresh cheese incorporated probiotic *Lactobacillus acidophilus* group lactic acid bacteria. Milchwissenschaft 60:167–171
19. Sperry MF, Silva HL, Balthazar CF, Esmerino EA, Verruck S, Prudencio ES, Nazzaro F (2018) Probiotic Minas Frescal cheese added with L. casei 01: Physicochemical and bioactivity characterization and effects on hematological/biochemical parameters of hypertensive overweighted women–A randomized double-blind pilot trial. J Funct Foods 45:435–443
20. Buriti FCA, da Rocha JS, Assis EG, Saad SMI (2005) Probiotic potential of Minas fresh cheese prepared with the addition of *Lactobacillus paracasei*. LWT Food Sci Technol 38: 173–180
21. Souza CHB, Saad SMI (2009) Viability of *Lactobacillus acidophilus* La-5 added solely or in co-culture with a yoghurt starter culture and implications on physico-chemical and related properties of Minas fresh cheese during storage. LWT Food Sci Technol 42:633–640
22. Roy D, Mainville I, Mondou F (1997) Selective enumeration and survival of bifidobacteria in fresh cheese. Int Dairy J 7:785–793
23. Burns P, Patrignani F, Serrazanetti D, Vinderola GC, Reinheimer JA, Lanciotti R, Guerzoni ME (2008) Probiotic crescenza cheese containing *Lactobacillus casei* and *Lactobacillus acidophilus* manufactured with high-pressure homogenized milk. J Dairy Sci 91:500–512
24. Kılıç GB, Kuleasan H, Eralp I, Karahan AG (2009) Manufacture of Turkish Beyaz cheese added with probiotic strains. LWT Food Sci Technol 42:1003–1008
25. Ryhänen EL, Pihlanto-Leppälä A, Pahkala E (2001) A new type of ripened, low-fat cheese with bioactive properties. Int Dairy J 11:441–447
26. Kasımoglu A, Göncüoglu M, Akgün S (2004) Probiotic white cheese with *Lactobacillus acidophilus*. Int Dairy J 14:1067–1073
27. Montoya D, Boylston TD, Mendonca A (2009) Preliminary screening of Bifidobacteria spp. and *Pediococcus acidilactici* in a Swiss cheese curd slurry model system: Impact on microbial viability and flavor characteristics. Int Dairy J 19:605–611
28. Yilmaztekin M, Ozer BH, Atasoy F (2004) Survival of *Lactobacillus acidophilus* LA-5 and *Bifidobacterium bifidum* BB-02 in white-brined cheese. Int J Food Sci Nutr 55:53–60
29. Bergamini CV, Hynes ER, Quiberoni A, Suárez VB, Zalazar CA (2005) Probiotic bacteria as adjunct starters: influence of the addition

methodology on their survival in a semi-hard Argentinean cheese. Food Res Int 38:597–604

30. Bergamini CV, Hynes ER, Zalazar CA (2006) Influence of probiotic bacteria on the proteolysis profile of a semi-hard cheese. Int Dairy J 16:856–866
31. Bergamini CV, Hynes ER, Palma SB, Sabbag NG, Zalazar CA (2009) Proteolytic activity of three probiotic strains in semi-hard cheese as single and mixed cultures: *Lactobacillus acidophilus*, *Lactobacillus paracasei* and *Bifidobacterium lactis*. Int Dairy J 19:467–475
32. Kourkoutas Y, Bosnea L, Taboukos S, Baras C, Lambrou D, Kanellaki M (2006) Probiotic cheese production using *Lactobacillus casei* cells immobilized on fruit pieces. J Dairy Sci 89:1439–1451
33. Albenzio M, Santillo A, Marino R, Della Malva A, Caroprese M, Sevi A (2015) Identification of peptides in functional Scamorza ovine milk cheese. J Dairy Sci 98:8428–8432
34. Phillips M, Kailasapathy K, Tran L (2006) Viability of commercial probiotic cultures (*L. acidophilus*, *Bifidobacterium* sp., *L. casei*, *L. paracasei* and *L. rhamnosus*) in Cheddar cheese. Int J Food Microbiol 108:276–280
35. Vinderola CG, Mocchiutti P, Reinheimer J, A. (2002) Interactions among lactic acid starter and probiotic bacteria used for fermented dairy products. J Dairy Sci 85:721–729
36. Gomes AMP, Malcata FX (1998) Development of probiotic cheese manufactured from goat milk: response surface analysis via technological manipulation. J Dairy Sci 81:1492–1507
37. Etchepare MDA, Raddatz GC, Cichoski AJ, Flores ÉMM, Barin JS, Queiroz Zepka L (2016) Effect of resistant starch (Hi-maize) on the survival of Lactobacillus acidophilus microencapsulated with sodium alginate. J Funct Foods 21:321–329
38. Mirzaei H, Pourjafar H, Homayouni A (2012) Effect of calcium alginate and resistant starch microencapsulation on the survival rate of lactobacillus acidophilus La5 and sensory properties in Iranian white brined cheese. Food Chem 132:1966–1970
39. Santillo A, Albenzio M, Bevilacqua A, Corbo MR, Sevi A (2012) Encapsulation of probiotic bacteria in lamb rennet paste: effects on the quality of Pecorino cheese. J Dairy Sci 95: 3489–3500
40. Figliola L, Santillo A, Giovanna MC, Caroprese M, Albenzio M (2021) Nonbovine milk products as probiotic and prebiotic food. In: Cruz AG, Ranadheera CS, Nazzaro F, Mortazavian A (eds) Probiotics and Prebiotics in Foods: challenges, innovations, and advances. Academic Press: UK. ch 7,, pp 115–133
41. Santillo A, Albenzio M (2015) Sensory profile and consumers' liking of functional ovine cheese. Foods 4:665–677
42. Santillo A, Albenzio M (2008) Influence of lamb rennet paste containing probiotic on proteolysis and rheological properties of pecorino cheese. J Dairy Sci 91:1733–1742
43. Santillo A, Albenzio M, Quinto M, Caroprese M, Marino R, Sevi A (2009) Probiotic in lamb rennet paste enhances rennet lipolytic activity and conjugated linoleic acid and linoleic acid content in Pecorino cheese. J Dairy Sci 92:1330–1337
44. Sultana K, Godward G, Reynolds N, Arumugaswamy R, Peiris P, Kailasapathy K (2000) Encapsulation of probiotic bacteria with alginate-starch and evaluation of survival in simulated gastro-intestinal conditions and in yoghurt. Int J Food Microbiol 62:47–55
45. Anal AK, Stevens WF, Remuñán-López C (2006) Ionotropic cross-linked chitosan microspheres for release of ampicillin. Int J Pharmacol 312:166–173
46. Homayouni A, Ansari F, Azizi A, Pourjafar H, Madadi M (2020) Cheese as a potential food carrier to deliver probiotic microorganisms into the human gut: A review. Curr Nutr Food Sci 16:15–28
47. Reale A, Di Renzo T, Coppola R (2019) Factors affecting viability of selected probiotics during cheese-making of pasta filata dairy products obtained by direct-to-vat inoculation system. LWT Food Sci Technol 116:108476
48. Soares MB, Almada CN, Almada CN, Martinez RCR, Pereira EPR, Balthazar CF, Cruz AG, Ranadheera CS, Sant'Ana AS (2019) Behavior of different Bacillus strains with claimed probiotic properties throughout processed cheese ("requeijão cremoso") manufacturing and storage. Int J Food Microbiol 307:108288
49. Champagne CP, Gagnon D, St-Gelais D, Vuillemard JC (2009) Interactions between *Lactococcus lactis* and *Streptococcus thermophilus* strains in Cheddar cheese processing conditions. Int Dairy J 19:669–674
50. Gagné G (2012) Viabilité de souches probiotiques commerciales au cours de la fabrication et de l'affinage du fromage cheddar, Master thesis, Laval University. Viabilité de souches probiotiques commerciales au cours de la fabrication et de l'affinage du fromage cheddar (ulaval.ca)

51. Siró I, Kápolna E, Kápolna B, Lugasi A (2008) Functional food. Product development, marketing and consumer acceptance—A review. Appetite 51:456–467
52. Cuffia F, George G, Renzulli P, Reinheimer J, Meinardi C, Burns P (2017) Technological challenges in the production of a probiotic pasta filata soft cheese. LWT Food Sci Technol 81:111–117
53. Kemp DSE, Hollowood DT, Hort DJ (2009) Requirements for sensory testing sensory evaluation. pp 30-65
54. Demers-Mathieu V, St-Gelais D, Audy J, Laurin E, Fliss I (2016) Effect of the low-fat Cheddar cheese manufacturing process on the viability of Bifidobacterium animalis subsp. lactis, Lactobacillus rhamnosus, Lactobacillus paracasei/ casei, and Lactobacillus plantarum isolates. J Funct Foods 24:327–337
55. Leeuwendaal NK, Hayes JJ, Stanton C, O'Toole PW, Beresford TP (2022) Protection of candidate probiotic lactobacilli by Cheddar cheese matrix during simulated gastrointestinal digestion. J Funct Foods 92:105042
56. Martins A, Deliza IBA, dos Santos R, Walter KMO, Martins EHM, Rosenthal JM (2018) Viability of probiotics in goat cheese during storage and under simulated gastrointestinal conditions. Food Bioproc Technol 11:853–863
57. Radulović Z, Cinović JM, Mirković N, Mirković M, Paunović DS, Ivanović M, Seratlić S (2017) Survival of spray-dried and free-cells of potential probiotic *Lactobacillus plantarum* 564 in soft goat cheese. Anim Sci J 88:1849–1854
58. Perotti MC, Wolf IV, Addis M, Comunian R, Paba A, Meinardi CA (2014) Incorporation of probiotic bacteria (Lactobacillus acidophilus and Bifidobacterium spp.) in Argentinean ovine cheese. J Dairy Sci Technol 94:255–267
59. Angelopoulou A, Alexandraki V, Georgalaki M, Anastasiou R, Manolopoulou E, Tsakalidou E, Papadimitriou K (2017) Production of probiotic Feta cheese using *Propionibacterium freudenreichii* subsp. *shermanii* as adjunct. Int Dairy J 66:135–139
60. Moraes AV, Pereira MO, Moraes KN, Rodrigues-Soares JP, Jesus GFA, Jatoba A (2018) Autochthonous probiotic as growth promoter and immunomodulator for Astyanax bimaculatus cultured in water recirculation system. Aquac Res 49(8):2808
61. Rodrigues R, Guerra G, Soaresc J, Santos K, Rolim F, Assis P, Araújo D, de Araújo Júnior RF, Garcia VB, de Araújo AA, Queiroga R (2018) Lactobacillus rhamnosus EM1107 in goat milk matrix modulates intestinal inflammation involving NF-κB p65 and SOCs-1 in an acidinduced colitis model. J Funct Food 50: 78–92
62. Albenzio M, Santillo A, Caroprese M, Marino R, Trani A, Faccia M (2010) Biochemical patterns in ovine cheese: influence of probiotic strains. J Dairy Sci 93:3487–3496
63. Corbo MR, Albenzio M, De Angelis M, Sevi A, Gobbetti M (2001) Microbiological and biochemical properties of Canestrato Pugliese hard cheese supplemented with Bifidobacteria. J Dairy Sci 84:551–561

Chapter 3

Probiotic Ice Creams

Vanessa Cortina Zanetti, Celso Fasura Balthazar, Callebe Camelo-Silva, and Silvani Verruck

Abstract

The growing consumer demand for healthier and more functional foods has led to the introduction of new ingredients in ice cream formulations with nutritional and physiological properties, such as probiotics. Incorporating probiotic bacteria into an ice cream should not affect the quality of the product. Therefore, its quality parameters such as air incorporation, melting rate, and sensory characteristics must be the same or better when compared to conventional ice cream. This chapter is a practical guidance for probiotic ice cream manufacture, presenting the steps and amount of probiotic addition into ice cream production.

Key words Ice cream, Probiotic, Functional food, Health

1 Introduction

Dairy products such as ice cream and frozen desserts can serve as vehicles for delivering probiotics to humans. In addition, ice cream can be kept in storage for longer time than other dairy products [9]. In this context, consumers' perception of healthy and functional foods led to the introduction, in the manufacture of ice cream, of ingredients with nutritional and physiological properties, such as probiotics [2, 24], dietary fibers [6–8], and synbiotics [9]. Ice cream is a complex colloidal system consisting of air cells, ice crystals, and partially destabilized fat globules dispersed in a continuous aqueous phase within polysaccharides, lactose, sugars and mineral salts [15]. As well the high level of total solids in ice cream provides protection to probiotic bacteria [20].

Probiotic ice cream is an acidified dairy frozen dessert of partially frozen structures. Acidification of the ice cream mixture can be carried out through direct inoculation of probiotic cultures, for example, *Bifidobacterium* spp. and *Lactobacillus* spp., the mixture of acidified milk or probiotic yogurt mixed with ice cream [3]. The therapeutic value of probiotic bacteria usually depends on the

Adriano Gomes da Cruz et al. (eds.), *Probiotic Foods and Beverages: Technologies and Protocols*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-3187-4_3,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2023

viability of these bacteria. Therefore, the International Dairy Federation [21] suggested that a minimum of 7 $\log_{10}$ probiotic bacterial colony forming units should be viable at the time of consumption per gram of the product (CFU/g), which is in accordance with the latest International Scientific Association for Probiotics and Prebiotics consensus statement [19]. The viability of probiotic bacteria in frozen dairy desserts is limited due to intrinsic environmental parameters, such as high redox value, oxygen toxicity, rupture of bacterial cell membranes during the freezing process, and the vulnerability of bacteria to acidic conditions [10]. Therefore, the efficiency in adding the probiotic depends on the inoculated dose, temperature, type of dairy foods, and the presence of air, and its viability must be maintained throughout the shelf life and intestinal environment, in addition to resisting at gastric pH [9].

Akin et al. [2] investigated the effects of inulin on the viability of probiotic bacteria in ice cream and found that their survival was higher in samples with inulin, probably due to the effect of the prebiotic. *L. acidophilus* and *B. lactis* counts were less than 5 $\log_{10}$ CFU/g in the control samples, while in the inulin-supplemented samples they were 5 $\log_{10}$ CFU/g. These results suggested that the addition of inulin stimulated the growth and improved the viability of those probiotic bacteria. Indeed, similar results were also verified by Balthazar et al. [9] in fermented sheep milk ice cream, in which synbiotic ice cream presented 7.61 $\log_{10}$ CFU/g and 5.18 $\log_{10}$ CFU/g against 6.89 $\log_{10}$ CFU/g and 5.02 $\log_{10}$ CFU/g from probiotic version viability after 150 days of frozen storage and in vitro simulated digestion, respectively, explained by inulin protection during storage and in vitro simulated digestion.

Homayouni et al. [20] verified in ice cream with microencapsulated or non-microencapsulated probiotics that there was a loss of only 0.7 and 0.4 $\log_{10}$ CFU/g of *Lacticaseibacillus casei* and *B. lactis* in the free state, respectively, and 0.3 and 0.2 $\log_{10}$ CFU/-gin the encapsulated state during the first month of storage. In the following five months, probiotic counts remained with a loss of 2.7 and 2.5 $\log_{10}$ CFU/g for the free state and 1.1 and 0.5 $\log_{10}$ CFU/g for the encapsulated state, and after the sixth month, the final drop was 3 0.4 and 2.9 $\log_{10}$ CFU/g in the free state of *L. casei* and *B. lactis*, respectively, against 1.4 and 0.7 $\log_{10}$ CFU/g in the encapsulated state. The numbers of viable probiotic bacteria in all types of ice cream were between 8 and 9 $\log_{10}$ CFU/g after three months of storage, the normal shelf life of ice cream. Pandiyan et al. [22] noticed that the melting rate of probiotic and symbiotic ice cream is faster and this behavior was attributed to the technological characteristics of the product such as freezing point and viscosity. This chapter is a practical guidance for probiotic ice cream manufacture, presenting the steps and amount of probiotic addition into ice cream production.

2 Materials

2.1 Ice Cream Ingredients

Ice cream can be processed with a variety of ingredients, including:

- Milk.
- Yogurt (*see* **Note 1**).
- Fat (*see* **Note 2**).
- Protein (*see* **Note 3**).
- Milk solids-not-fat (*see* **Note 4**).
- Water.
- Sweeteners (*see* **Note 5**).
- Stabilizers (*see* **Note 6**).
- Emulsifiers (*see* **Note 7**).
- Flavoring (*see* **Note 8**).
- Coloring.
- Probiotics—examples Table 1.
- Fruits.
- Nuts.
- Bakery pieces.
- Candy pieces.

Table 1 shows a traditional ice cream formulation. This formulation can be adapted with higher or lower values than those shown in Table 1, in addition to the addition of others, for specific purposes.

2.2 Ice Cream Equipment

The main equipment needed to produce ice cream are described below, along with suggestions for equipment to be purchased.

- **Doser:** Ingredient Doser A3 (Tetra Pak).
- **Mixer:** High shear blender (Bredoo Likwifier); High shear mixer B200–300 A (Tetra Pak).
- **Pasteurizer:** Pasteurizer D (Tetra Pak).
- **Homogenizer:** Industrial five-piston homogenizer (Tetra Pak); Homogenizer 250 (Tetra Pak).
- **Maturator:** Incubator (cooled Incubator ILW 115, POL-EKO-APARATURA.
- **Freezer:** Tetra Hoyer Frigus-KF freezer (TetraPak), WCB Ice Cream freezer (WCB Ice Cream); Ice Cream Machine (Delonghi, Il gelato, ICK5000), Ice Cream Machine (−5 °C; L/30–3, SEVEL Cooling INC.); Continuous Freezer S300 M2 (Tetra Pak).

Table 1
Traditional ice cream composition

Composition (%)	Amount (%)
Milk solids-not-fat	9.0–11.0
Milk fat	10.0–16.0
Sucrose	10.0–16.0
Corn syrup solids	2.0–5.0
Stabilizer	0.15–0.35
Emulsifier	0.10–0.15
Total solids	36.0–42.0
Water	58.0–64.0

Source: Adapted from [7]

- **Filler:** Rotary-type filler for cups and round nested containers (Huhtamaki, Inc.); In-line filler for square-round packages (TD Sawvel Co., Inc.); Ice Cream Smart Filler A1 (Tetra Pak).
- **Wrapper:** Ice Cream Wrapper A2 (Tetra Pak).
- **Hardening:** Super Deep Chest Freezer LY450LD (−35 °C) (Snow-MY).

2.3 Probiotic Strains

Inoculation of probiotics can be performed by Direct Vat Set (DVS) or by Propagation. The DVS method promotes the use of standardized freeze-dried cultures, with low amounts sufficient for inoculation. The propagation method is carried out by cell cultivation in a specific medium, purification of the culture, and subsequent incorporation into the product. Table 2 presents the species of probiotics used in the preparation of ice cream and describes the inoculation method used.

3 Methods

The processing of ice cream is divided into two distinct stages, the production of the mixture and the freezing operations. To produce quality ice cream, the steps must be carried out in a controlled manner, adapting the desired final characteristics. The elaboration steps include mixing, heat treatment, homogenization, maturation, freezing, packaging, and hardening (*see* Fig. 1).

1. Mix the ingredients, as described in Table 1, in a tank with agitation and heating, heating them to 50 °C to facilitate solubilization (*see* **Note 9**) [12].
2. After the complete incorporation of the ingredients, pasteurize the mixture at 70–85 °C from 30 s to 30 min (*see* **Note 10**) [5].

Table 2
Information about probiotic strains added to ice cream formulations

Probiotic strain	Inoculation dose (CFU/mL)	Inoculation method	Inoculation temperature (°C)	Storage (days)	Probiotic viability after storage (CFU/mL)	Reference
Lactobacillus acidophilus La-5®	9.0	90% of the milk was transferred into two sterile jars, and the milk samples were inoculated with freeze-dried *L. acidophillus* La-5® and incubated at 37 °C/4 hours until the pH reached 4.7.	4.0	60	7.37	[4]
Bifidobacterium bifidum	7.56–7.60	Freeze-dried *Bifidobacteria* was prepared in 200 mL of reconstituted skimmed milk (10% w/w), and it was incubated at 37 °C/24 hours. The probiotic was reactivated into 300 mL of milk and incubated at 37 °C until the pH became 4.6.	4.0	60	6.20–6.28	[5]
Lactobacillus acidophilus La-5®	8.20	The *L. acidophilus* La-5 was inoculated in the ice cream mix and incubated at 37 °C until the pH reached 5.80.	37	60	7.25	[13]
Lactiplantibacillus plantarum subsp. *plantarum* ATCC 8014	6.0–7.0	About 1% of *L. plantarum* subsp. *plantarum* inoculum was cultured in sterile man, Rogosa, and sharp broth and incubated overnight at 37 °C. after that, the cells were centrifugated and washed twice with sterile peptone water. Approximately 1 mL of *L. plantarum* subsp. *plantarum* after 20 h of incubation was added to 150 mL of pasteurized full cream milk.	40	60	>7.46	[16]
Saccharomyces boulardii *Lacticaseibacillus rhamnosus* GG	7.34 10.11	The *S. boulardii* was incubated in YPD broth for 48 h, and the *L. rhamnosus* was incubated in MRS broth for 24 h at 37 °C. both strains were grown in 5 L volumes to obtain them at desired levels before the inoculation of the ice cream	37	28	6.2 9.2	[26]

(continued)

Table 2
(continued)

Probiotic strain	Inoculation dose (CFU/mL)	Inoculation method	Inoculation temperature (°C)	Storage (days)	Probiotic viability after storage (CFU/mL)	Reference
		mix. After the incubation, the cultures were centrifuged, and obtained pellets were washed twice with PBS and inoculated to the pasteurized ice cream mix to 37 °C before and after the aging steps.				
Lacticaseibacillus paracasei subsp. *Paracasei* L-26 *Lacticaseibacillus casei* 431 *Lactobacillus acidophilus* La-5®	9.0	The starter probiotic cultures were previously inoculated in milk at 40 °C for 5 hours. 5% of inoculum was added to the ice mixes.	37	21	10.18–10.17 9.88–9.85 8.83–9.68	[17]
Bifidobacterium lactis (Bl-04)	9.0	The culture was dissolved into UHT milk and activated to obtain 10^9 CFU/mL of bacteria cells in MRS broth.	2	120	7.16	[14]
Lacticaseibacillus paracasei subsp. *Paracasei* L-26 *Bifidobacterium longum* + *Bifidobacterium bifidum* B-94	8.0–9.0	The probiotic cultures were inoculated in the ice cream mix by the commercial company's recommendations. The inoculated mixtures were left to incubate at 37 °C, which was carried out until the pH values reached 4.8–4.9.	37	120	8.19 8.15	[1]
Lactiplantibacillus plantarum subsp. *plantarum* LP299v *Lacticaseibacillus casei* ATCC 393	8.50–9.0	An aliquot of each probiotic culture was individually transferred into MRS broth and placed in an incubator at 37 °C for 24 hours. After, the 10 mL culture was diluted to 100 mL and re-incubated at 37 °C/48 h. it was centrifuged to isolate the probiotics from the MRS.	37	60	>7.50	[25]

Lactobacillus acidophilus *Bifidobacterium lactis*	8.0	Freeze-dried cultures of probiotic strains were inoculated separately in glass tubes containing MRS broth and incubated at 37 °C/24 h under aerobic conditions. Then they were centrifuged, and washed twice with sterile saline. The resulting pellet was diluted in sterile saline.	37	180	5.95 >6.0	[18]
Lacticaseibacillus casei 01	6.0	100 mg (w/w) of freeze driedLactobacillus casei-01 in 1 L (v/v) of skimmed sheep milk (w/v) for 6-h incubation. Subsequently, fermented sheep milk was added by sheep milk fat and skimmed sheep milk to totalize 2 L of mix added with inulin, sugar, and stabilizer/emulsifier.	37.0	150	> 7.0	[9]

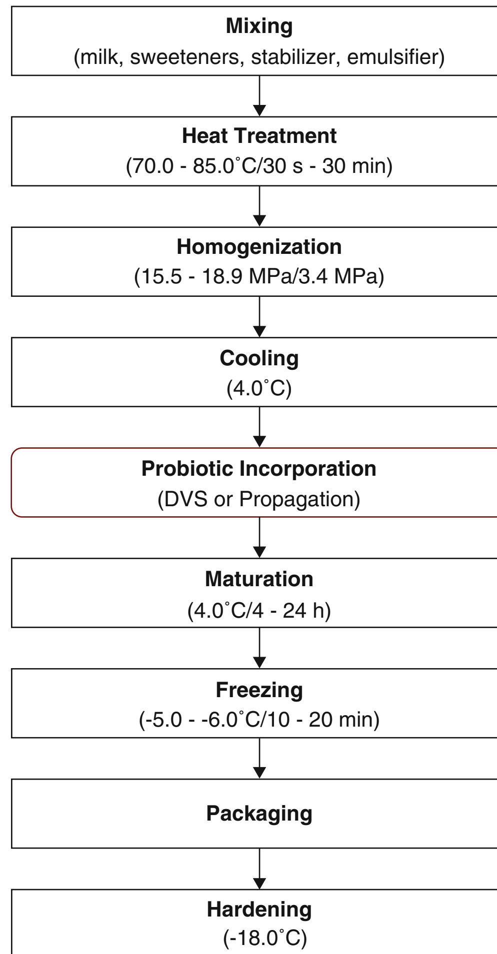

Fig. 1 Flowchart of ice cream making

3. Then homogenize the pasteurized mixture in two steps: at high pressure (15.5–18.9 MPa) and after low pressure (3.4 MPa) (*see* **Note 11**) [23].
4. After homogenization, incorporate probiotic microorganisms into the mixture (*see* **Note 12**) and cool until you reach 4 °C to start the maturation stage.
5. Perform maturation by stirring the mixture at 4 °C temperature for a time of 4–24 h (*see* **Note 13**). After complete maturation, the mixture proceeds to freeze.
6. Freeze the ice cream in equipment containing a rotary stirrer, for air incorporation, up to an overrun of 50%, at a temperature of −5 to −6 °C for 10–20 min (*see* **Notes 14, 15,** and **16**).
7. Then fill the ice cream and proceed to the final hardening at −30 °C or lower (*see* **Note 17**), with subsequent storage at −18 °C (*see* **Note 18**).

4 Notes

1. Yogurt is used in the preparation of frozen yogurt.
2. Fat sources can come from milk fat, such as cream milk, butter, and butter oil, fats, and oils from plants, such as corn, sunflower, canola, and peanut, and blends of oils [15].
3. Protein sources may or may not be milk. Whey proteins and caseins are used, in addition to soy proteins and nuts [15]. Proteins are also used to give ice structuring.
4. The milk solids-not-fat contribute to the flavor and texture. The industry usually uses concentrated milks, dried skim and whole milk, milk power blends, and whey products [15].
5. Sweetening sources may be corn sweeteners, maple sugar, honey, invert sugar, fructose, molasses, malt syrup, brown sugar, lactose, sugar alcohols, sorbitol, mannitol, xylitol, and other nonnutritive sweeteners such as saccharin, aspartame, and sucralose [15].
6. Examples of stabilizers used: carob gum, guar gum, xanthan gum, sodium carboxymethylcellulose, sodium alginate, microcrystalline cellulose, carrageenan, gelatin, and pectin [23].
7. The emulsifiers added in ice cream formulations are of two types: mono- and diglycerides and sorbitan esters, as polysorbate 80. Some factories also use eggs or egg yolk.
8. The most used flavors are chocolate, vanilla, and strawberry. But, neopolitan, lemon, nut, pear, rum and raisin, cookies and cream, and others can also be added.
9. Automatic dosing pumps or tanks in load cells can add liquid ingredients. Dry ingredients are added by pumping at high speed or with high shear mixers to prevent the formation of lumps. Dry ingredients should be incorporated into the mixture at a temperature below 30 °C [12, 15].
10. The four methods to pasteurize ice cream can be low-temperature long-time (LTLT—69 °C/30 min), high-temperature short-time (HTST—83 °C/15 s), higher-heat shorter-time (HHST—90 °C/1–3 s), or ultra-high temperature (UHT—138 °C/$\geq$2 s) [11]. Batch pasteurization uses double-shirt tanks, in which the mixture is heated, with steam circulation or hot water inside, performing heat changes. Continuous pasteurization is performed in heat exchangers, and there may be a preheating of the mixture between 30 and 40 °C to mix the ingredients.
11. Homogenization is performed in two stages so that in the first stage fat globules tend to group and form agglutinated. In the second stage, the adsorption of proteins occurs on the fat surface, avoiding further regrouping, and making the emulsion more stable.

12. The cultures can be added to ice cream in several ways, of type DVS (direct vat set), for direct addition of the product in a pasteurized mixture, or in the use of milk as a substrate for fermentation [4].

    The freeze-dried probiotic culture can be added to the mixture before maturation and freezing, which presents advantages related to the easy insertion of the same in the mixture; however, as it is not in its active form, it may be that the probiotic remains inactive. In addition to freeze-dried culture, there is the possibility of incorporating probiotic biomass into the mixture before maturation [5]. The added biomass ferments the mixture, which then proceeds to maturation.

    When milk is used as substrate for probiotic incorporation, the step can be performed by means of a partial mixture of milk with probiotic, so that 10 to 30% of milk proceeds to a fermentation stage with probiotic culture, at 37 °C, for up to 12 hours in anaerobiosis, and then that fermented milk is incorporated into the rest of the mixture before freezing. This partial fermentation of milk promotes the activation of probiotic culture, besides not significantly altering the organoleptic characteristics of ice cream [4].

    Also, from the use of milk, it can be entirely fermented by probiotic culture and, after, the other ingredients are added. However, this type of process ends up resulting in more acidic sensory characteristics due to the high production of lactic acid [4].

13. In the maturation stage, the addition of flavorings and dyes that are sensitive to the heat of thermal processing is carried out aseptically. The additives added, both in the mixing stage and in maturation, cannot interfere with the action of the probiotic, nor cause any kind of damage. At this stage occurs the development of the sensory characteristics of flavor and aroma of the product.

14. The freezing stage is one of the main parts of the preparation of ice cream because there is the incorporation of air in the ice cream, also known as overrun, a step that gives the characteristic of the body and texture of the ice cream. The amount of overrun should be between 2.5 to 3 times the total solids of the ice cream. The presence of this stage in the preparation of ice cream causes risks to the survival of probiotics, due to oxygen toxicity, and the use of aerotolerant strains is necessary. In addition, the size of the particles should be monitored, and the ice crystals should have sizes of 30 to 50 um, air bubbles from 20 to 80 um, the agglomerated fat globules from 2 to 20 um, and the isolated fat globules of 0.1 to 15 um [23].

15. The freezing step takes place in two stages. The first step takes place by passing the mixture in a high-beat shaved surface heat exchanger to allow extensive nucleation of ice crystals and air incorporation. The second step is freezing the ice cream packed in reduced time to prevent the formation of large crystals [23].
16. The probiotic cultures are usually sensitive to freezing, so the incorporation of air and the reduction at shallow temperatures become a lethal medium for these bacteria. The use of cryo-protection, such as sugars, fats, or proteins, promotes the improvement of the resistance of these bacteria to the frozen environment, considering freezing time. The encapsulation of probiotics with these cryoprotectants, in addition to protecting against freezing, also has improved viability during passage in the gastrointestinal tract.
17. During storage, it is extremely important that there is no great variation in temperature, to the point of releasing water in the ice cream, because this failure promotes recrystallization, with the increase of ice crystals, leading to an unpleasant texture in the product. In ice cream, small and quite numerous crystals are sought, the opposite of this is considered a manufacturing defect [23].
18. The formation of ice crystals occurs in two stages. Nucleation is the step that occurs on the wall of the heat exchanger, with small and numerous ice crystals. The low temperature during hardening promotes the continuous growth of the formed crystals. When hardening is slow, the remaining water in the ice cream migrates to the crystals already formed, causing large crystals, which promote the disruption of the cell membrane of probiotics and lead to their inactivation [23].

References

1. Acu M, Kinik O, Yerlikaya O (2020) Probiotic viability, viscosity, hardness properties and sensorial quality of synbiotic ice creams produced from goat's milk. Food Sci Technol 41(1): 167–173. https://doi.org/10.1590/FST.39419
2. Akın MB, Akın MS, Kırmacı Z (2007) Effects of inulin and sugar levels on the viability of yogurt and probiotic bacteria and the physical and sensory characteristics in probiotic ice-cream. Food Chem 104:93–99
3. Alamprese C, Foschino R, Rossi M, Pompei C, Corti S (2005) Effects of lactobacillus rhamnosus CG addition in ice cream. Int J Dairy Technol 58:200–206
4. Arslaner A, Salik MA (2022) Probiotic ice cream with Malus floribunda fruit sauce: quality properties, mineral, and volatile composition. J Food Proc Preserv 46(5):e16566. https://doi.org/10.1111/JFPP.16566
5. Atallah AA, Ismail EA, Yehia HM, Elkhadragy MF, Aloufi AS, Gemiel DG (2022) Physicochemical, microbiological and microstructural characteristics of sucrose-free probiotic-frozen yogurt during storage. Foods 11(8). https://doi.org/10.3390/foods11081099
6. Balthazar CF, Silva HLA, Celeguini RMS, Santos R, Pastore GM, Conte-Júnior CA, Freitas MQ, Nogueira LC, Silva MC, Cruz AG (2015) Effect of galactooligosaccharide addition on the physical, optical, and sensory acceptance of vanilla ice cream. J Dairy Sci 98:4266–4272. https://doi.org/10.3168/jds.2014-9018

7. Balthazar CF, Silva HLA, Moraes J, Vieira AH, Neto RPC, Granato D, Freitas MQ, Calado VMA, Tavares MIB, Cruz AG (2017) Assessing the effects of different prebiotic dietary oligosaccharides in sheep milk ice cream. Food Res Int 91:38–46. https://doi.org/10.1016/j.foodres.2016.11.008
8. Balthazar CF, Silva HLA, Cavalcanti RN, Esmerino EA, Cappato LP, Abud YKD, Moraes J, Andrade MM, Freitas MQ, Sant'Anna C, Raices RSL, Silva MC, Cruz AG (2017) Prebiotics addition in sheep milk ice cream: A rheological, microstructural and sensory study. J Funct Foods 35:564–573. https://doi.org/10.1016/j.jff.2017.06.004
9. Balthazar CF, Silva HLA, Esmerino EA, Rocha RS, Moraes J, Carmo MAV, Azevedo L, Camps I, Abud YKD, Sant'Anna C, Franco RM, Freitas MQ, Silva MC, Raices RSL, Escher GB, Granato D, Ranadheera CS, Nazarro F, Cruz AG (2018) The addition of inulin and lactobacillus casei 01 in sheep milk ice cream. Food Chem 246:464–472. https://doi.org/10.1016/j.foodchem.2017.12.002
10. Cruz AG, Antunes AEC, Sousa ALOP, Faria JAF, Saad SMI (2009) Ice cream as a probiotic food carrier. Food Res Int 42:1233–1239
11. Cruz AG et al. (2017) Processamento de produtos lácteos: Queijos, Leites Fermentados, Bebidas Lácteas, Sorvete, Manteiga, Creme de Leite, Doce de Leite, Soro em Pó e Lácteos Funcionais. 1st edn. Rio de Janeio: Elsevier, 2017
12. Deosarkar SS, Khedkar CD, Kalyankar SD, Sarode AR (2015) Ice cream: uses and method of manufacture. In: Encyclopedia of food and health. Elsevier Inc., pp 391–397. https://doi.org/10.1016/B978-0-12-384947-2.00384-6
13. Elkot WF, Ateteallah AH, Al-Moalem MH, Shahein MR, Alblihed MA, Abdo W, Elmahallawy EK (2022) Functional, physicochemical, rheological, microbiological, and organoleptic properties of Synbiotic ice cream produced from camel Milk using black Rice powder and lactobacillus acidophilus LA-5. Fermentation 8(4). https://doi.org/10.3390/fermentation8040187
14. El-Sayed HS, Salama HH, El-Sayed SM (2015) Production of synbiotic ice cream. Int J Chem Tech Res 1(7):138–147
15. Goff HD, Hartel RW (2013) Ice cream, 7th edn. Springer, New York, p 462. Innocente, N., Biasutti, M., Venir, E., Spaziani, M., & Marchesini, G. (2009). Effect of high-pressure homogenization on droplet size distribution and rheological properties of ice cream mixes. J Dairy Sci, 92, 1864–1875
16. Goktas H, Dikmen H, Bekiroglu H, Cebi N, Dertli E, Sagdic O (2022) Characteristics of functional ice cream produced with probiotic saccharomyces boulardii in combination with lactobacillus rhamnosus GG. LWT 153: 112489. https://doi.org/10.1016/J.LWT.2021.112489
17. Haghani S, Hadidi M, Pouramin S, Adinepour F, Hasiri Z, Moreno A, Munekata PES, Lorenzo JM (2021) Application of cornelian cherry (Cornus mas L.) Peel in probiotic ice cream: functionality and viability during storage. Antioxidants 2021 10:1777. https://doi.org/10.3390/ANTIOX10111777
18. Hayayumi-Valdivia M, Márquez-Villacorta LF, Pretell-Vásquez CC (2021) Effect of microencapsulation and mango peel powder on probiotics survival in ice cream. Brazilian J Food Technol 24:2021. https://doi.org/10.1590/1981-6723.30919
19. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B et al (2014) The international scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol 11:506–514
20. Homayouni A, Azizi A, Ehsani MR, Yarmand MS, Razavi SH (2008) Effect of microencapsulation and resistant starch on the probiotic survival and sensory properties of synbiotic ice cream. Food Chem 111:50–55
21. IDF (1992) International standard 163. General standard of identity for fermented milks. International Dairy Federation, Brussels
22. Pandiyan C, Annal Villi R, Kumaresan G, Murugan B, Gopalakrishnamurthy TR (2012) Development of synbiotic ice cream incorporating lactobacillus acidophilus and saccharomyces boulardii. Int Food Res J 19(3):1233–1239
23. Pimentel TC, de Oliveira LIG, de Souza RC, Magnani M (2022) Probiotic ice cream: A literature overview of the technological and sensory aspects and health properties. In: International journal of dairy technology (Vol. 75, issue 1, pp. 59–76). John Wiley and Sons Inc. https://doi.org/10.1111/1471-0307.12821
24. Ranadheera CS, Evans CA, Adams MC, Baines SK (2012) In vitro analysis of gastrointestinal tolerance and intestinal cell adhesion of probiotics in goat's milk ice cream and yogurt. Food Res Int 49:619–625
25. Sabet-Sarvestani N, Eskandari MH, Hosseini SMH, Niakousari M, Hashemi Gahruie H,

Khalesi M (2021) Production of synbiotic ice cream using lactobacillus casei/lactobacillus plantarum and fructooligosaccharides. J Food Process Preserv 45(5):e15423. https://doi.org/10.1111/JFPP.15423

26. Yerlikaya O, Ender G, Torunoglu FA, Akbulut N (2013) Production of probiotic milk drink containing *lactobacillus acidophilus*, *Bifidobacterium animalis* subsp. *lactis* and *lactobacillus casei*. Agrofood Ind 24(2):49–52

Chapter 4

Probiotic Butter

Patrícia Blumer Zacarchenco, Leila Maria Spadoti, Adriana Torres Silva e Alves, Vanessa Cortina Zanetti, and Silvani Verruck

Abstract

In recent years butter has seen a growth in its consumption because of the understanding of the physiological effects of dairy fat. The small-scale batch production of butter depends on the operator's hands-on skill and experience about cream aging, ending point of churning, and other parameters at each of the manufacturing stages. Large-scale processes are automated and allow better control to produce butter with constant characteristics. While the principles of butter making have not changed significantly over many decades, with greater understanding, control of key parameters during cream preparation and processing conditions have improved. In recent years there has been a lot of products from butter post-churning, mixing new ingredients like probiotics, flavors, and spices that will be beneficial in attracting and engaging consumers. Butter has shown to be a good matrix for adding probiotics and maintaining its viability throughout storage due to the fat protection effect. There are several ways of adding probiotics in butter, such as in microencapsulated form, together with traditional starters during cream fermentation or during the working step. Thus, this chapter is a practical guidance for probiotic butter manufacture.

Key words Milk fat, Butter, Probiotic, Functional food, Health

1 Introduction

The amount of fat in milk and the fatty acids content can be influenced by many parameters such as the diet of the cows, number of pregnancies, animal health, breed, and stage of lactation. The type of feed or pasture consumed by the animal has been used to modify the fatty acid profile of dairy fat to obtain beneficial nutrients as the increased polyunsaturated content. However, all changes in the percentage and types of fatty acids in milk can influence the processing and characteristics of manufactured products. The lipid fraction of milk corresponds to 4.2% of dairy solids and is composed of about 98% of triglycerides and other components in smaller amounts, such as phospholipids, sterols, lipopro-

Adriano Gomes da Cruz et al. (eds.), *Probiotic Foods and Beverages: Technologies and Protocols*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-3187-4_4,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2023

teins, and vitamins. It is important to remember that milk is a liquid emulsion of oil in water, which is very nutritious because it is a source of several essential compounds [1].

The butter production has grown worldwide [2] while the price of butter has doubled in the international market and its consumption grows by about 4% per year [3]. Around 40% of the consumers consulted on the research stated that they started to buy more butter because it is a healthier option [3]. This change in demand for butter is in function of changes in consumer perceptions of dairy fat. While in the past dairy fat has been associated with an increased risk of cardiovascular disease, there is growing evidence to suggest that regular consumption of dairy products with a regular fat content is not associated with an increased risk of these diseases [4]. In addition, some benefits of consuming dairy products with a regular fat content have been demonstrated in terms of the presence of bioactive nutrients and anti-inflammatory properties [5]. Butter is a complex of at least 400 types of fatty acids, the consumption of which, combined with a balanced diet and a healthy lifestyle, can protect against certain types of cancer, and reduce the risk of cardiovascular disease. Due to this new perspective on milk fat and its effect on human health, research in ruminant nutrition has generated results that demonstrate the production of bovine milk from tropical grasses can increase the level of beneficial fats. One of these fatty acids, known by the acronym CLA (conjugated linoleic acid), from its chemical name, has proven anti-carcinogenic properties. Several works carried out by [3] have been demonstrating that cows fed with fresh elephant grass increased the CLA content in the milk, therefore, also in the butter.

Butter is one of the oldest dairy products that is still being made. Butter is obtained by concentrating milk fat in the form of cream, which is then churned until the oil-in-water emulsion is converted to water-in-oil emulsion. There are criteria for the composition of the product classified as butter. According to Codex Alimentarius [6] butter is a fatty product derived exclusively from milk that must contain at least 80% fat, a maximum of 16% moisture, and 2% defatted milk solids (proteins, lactose, and minerals). USDA [7] also defines this minimum fat content, in addition to other parameters, and indicates a three-level classification (AA, A, and B) based on the sensory attributes of the butter. In Brazil, there are also standards defined in the legislation to regulate the minimum fat content in butter, in addition to the maximum moisture content, sodium chloride and acidity, among other physical–chemical and microbiological parameters [8, 9].

Butter can be produced from sweet cream or fermented cream, with and without salt. It is used in several culinary preparations, such as an ingredient in the chocolate, confectionery, and bakery industries, in addition to the dairy industry. In cream fermentation, different probiotic or non-probiotic lactic ferments can be used.

The ferment can be defined as a microbial preparation containing a large number of cells of at least one microorganism that is added to a raw material (in this case, cream) to produce the fermented food and it can speed up and control the fermentation process [10].

The group of lactic acid bacteria (LAB) plays an important role in this process and has a long history of safe application and consumption in fermented foods and beverages. These bacteria generate rapid acidification of the raw material through the production of organic acids, mainly lactic acid, but also acetic acid, ethanol, aromatic compounds, bacteriocins, exopolysaccharides, and several important enzymes. With this, these bacteria increase shelf life, improve texture, and contribute to pleasant sensory characteristics in the final product [10]. Most of the lactic acid bacteria belonged to the genus *Lactobacillus*, which was recently reclassified [11]. The member species were distributed in 25 genera, including those that remained in the original genus *Lactobacillus.* Thus, species of probiotic lactic acid bacteria, or not, applied in butter studies may have been reclassified to other genera and have their gender designation changed [12]. Several LAB strains are recognized as probiotics. Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [13] indicating that viability is a necessary requirement to ensure the expected benefits. In turn, paraprobiotics, defined as non-viable cells, and postbiotics, which are substances generated by bacteria, respectively [14–16], have been studied and the health benefits are not all necessarily related only to viable cells. Thus, probiotics, but also paraprobiotics and postbiotics, can be present in butter, adding additional beneficial characteristics to the bioactivity of naturally present nutrients such as CLA, phospholipids, and vitamins. In this sense, this chapter's purpose is to describe the butter manufacturing and possible steps where probiotics can be added during the process.

2 Material and Methods

There are different butter varieties mainly involving the presence or absence of salt and primary fermentation of the cream. The type of ferment used in this fermentation will influence the flavor of the final butter (*see* **Note 1**). There are also different methods of making butter which include emulsification and the more well-known churning method. Figure 1 shows the process of making salted butter from cream using churning method. In addition, the main stages in the manufacture of butter will be presented so it can be verified in which stages the probiotics can be added.

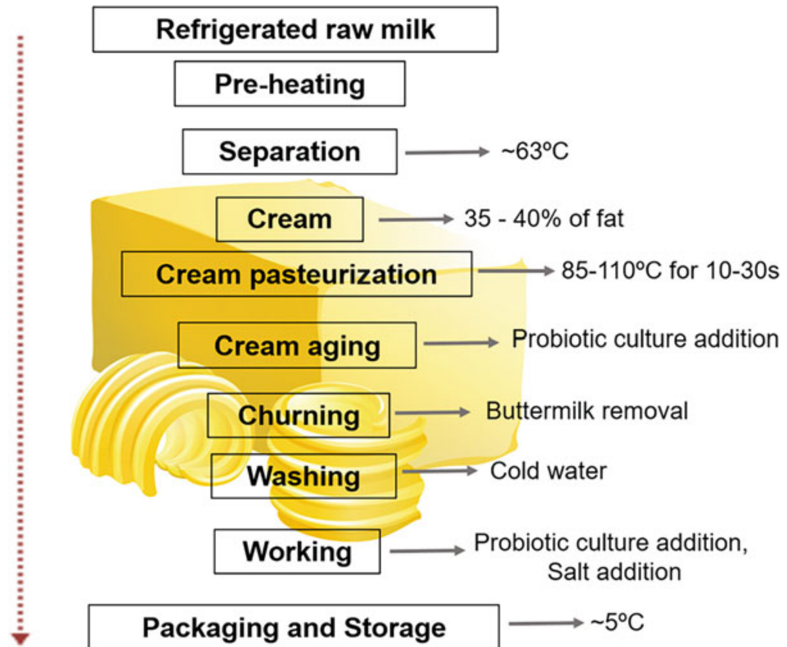

Fig. 1 Steps of the process of making salted butter

2.1 Cream Preparation

The cream can be extracted from milk kept at rest if it has not been treated in the double-piston homogenizer, as it separates naturally due to the fat particles being less dense and floating in the upper portion of the tank. It is more applied, however, the separation using centrifugation with the milk heated above 40 °C so that less damage occurs to the milk fat globule membrane (MFGM) since the fat will be in liquid form. An optimal temperature condition is 63 °C, for a cream with 35 to 40% fat or 45 to 48%, depending on the equipment and process [1]. There is a batch equipment called skimmer used to separate the cream from the milk and continuous separators for larger industrial volumes. After separating the cream, it undergoes thermal treatment in plate heat exchangers with time and temperature binomials between 85 and 110 °C for 10 to 30 seconds to eliminate pathogenic microorganisms and reduce the counts of deteriorating microorganisms (*see* Fig. 1). At this stage, vacuum can be applied to remove volatile compounds with unpleasant odors that can influence the sensory characteristics of the final product. After the thermal treatment, the cream is cooled to 4–5 °C so the maturation stage takes place during a minimum period of 4 hours or "overnight" when the milk fat crystallizes (*see* **Note 2**).

For the cream maturation, there are different combinations of time/temperature that influence the shape of the crystals that appear in the cream and it will affect characteristics as consistency and spreadability of the butter after churning. The greater efficiency of the different cream maturation time and temperature binomials

are related to factors such as the season of the year and the levels of unsaturated fatty acids present [17, 18] (*see* **Note 3**). Slow and rapid cooling of the cream can result in rheological properties and crystals with similar polymorphic forms (α- and β'-), but with differences in their microstructure (*see* **Note 4**). Unlike butter produced from slowly cooled cream, rapid cooling results in butter with more uniformly sized crystals (*see* **Note 5**). When the amount of low melting fat is reduced (iodine number less than 35 I_2/100g) the butter will have a firm texture if the cream is cooled in stages. In these cases, the application of the "cool/heat/cool" procedure called "Alnarp method" can be beneficial for the butter final texture. Cooling to 8 °C after heat treatment, keeping at this temperature for 2 hours, heating to 20 °C, keeping for 2 to 3 hours at this temperature, and finally cooling to room temperature (*see* **Note 6**). Finally, the cream is cooled down to the churning temperature. There are several temperature sequences for chilling the cream before churning to separate the butter grains. For example, a combination is cool to 8 °C, raise to 18 °C and cool to 12 °C or cool to 8 °C, raise to 22 °C and cool again to 12 °C (*see* **Note** 7).

One of the ways of adding probiotics to butter is through their direct inoculation into the cream with or without a fermentation step (*see* **Note 8**). Several probiotic strains are available for use as DVS (Direct vat set) inoculums in butter production. The main cultures already used in probiotic butter and cell viability after storage are summarized in Table 1. The cell amount to be added into the butter varies between different strains to ensure the health benefits. The amounts of cells added by [19, 20], and others are adequate to keep cells active until the end of storage.

2.2 Batch Butter Making Process

1. The pre-treated cream is transferred to the mixer (*see* **Note 9**) where it is stirred at high speed to break the emulsion, and the grains of butter emerge as the drained buttermilk separates.
2. The temperature range for churning is 8 to 12 °C.
3. After the emulsion has broken, the buttermilk is drained from the tank and can be filtered to remove fat lumps.
4. Add fresh water to the churner and whisk to wash the grains and remove buttermilk residues until the water runs clear.
5. The working (mechanical treatment) of the butter takes place at low speed with the tank valve open to drain the buttermilk expelled from it. When the amount of water is sufficiently low, the valve is closed and, if necessary, salt is added.
6. As the working time is longer in the batch process compared to the continuous one, the salting process can take place by dry, wet, or brine salting (*see* **Note 10**).
7. When the butter appears to be dry, the working is stopped and the moisture content is measured and adjusted, continuing the mechanical work until all the free water has been absorbed.
8. Then, the butter is packaged.

Table 1
Information about probiotic strains added to butter formulations

Probiotic strain	Inoculation dose (log CFU/g)	Inoculation method	Inoculation temperature (°C)	Storage (days)	Probiotic viability after storage (CFU/g)	Reference
Lactobacillus acidophilus La-14	10.63	The microorganisms were encapsulated using the technique of extrusion and ionic gelation. The encapsulating matrix used was low viscosity sodium alginate at a concentration of 10 g/L, dripped onto the covering material, 0.1 Mol/L calcium chloride dihydrate. The capsules had an average diameter of 1.8 mm. The culture was lyophilized.	10.0	60	8.90	[19]
Bifidobacterium animalis subsp. *lactis* ATCC 27536 *Lactobacillus acidophilus* ATCC 4356	8.98	The spray-dried formulation was prepared by a combination of probiotic microorganisms, in a conjugated whey protein hydrolysate (WPH10-maltodextrin) matrix (1:1). Pure freeze-dried probiotic cultures were obtained from ATCC. Fresh cultures were obtained after two transfers in de man, Rogosa, and Sharpe broth (MRS) supplemented with 0.05% (w/v) L-cysteine at 37 °C for 72 h, under anaerobic conditions. Propagation of the cultures was continued, and the cells were harvested in their late log phase by centrifugation at 7000 × g for 10 min at 4 °C. the cell pellets were washed twice in phosphate-buffered saline (PBS) and suspended to achieve cell suspensions of 10 log CFU/mL.	–	–	5.09–8.22	[20, 21]
Lacticaseibacillus casei *Lactiplantibacillus plantarum* subsp. *plantarum* *Lacticaseibacillus paracasei* subsp. *paracasei* Lyofast CPR1	8.2–8.7	The probiotic culture was pre-activated in pasteurized milk for 15 h at 35 °C, with an initial concentration of 0.5% (w/v) of milk. Then, the pre-activated inoculum, with a probiotic level of 8.2–8.7 log CFU/mL was used to inoculate the cream applied to produce probiotic butter samples.	19.0	1.42	>6.0	[22]

Lacticaseibacillus casei LAFTI L 26; CSL3	7.0	After obtaining butter, the formulations with probiotic bacteria were supplemented using 0.1% of each probiotic (7 log CFU/g of butter).	4.0	90	4.81–5.80	[23]
Lactobacillus acidophilus La-5	6.0–8.0	The culture was used for fermenting cream. It was added to the cream at fermentation temperature, which varied according to the characteristics of the seasons. On I group the fermentation took place at 30 °C and physical maturing at 7 °C. on II group the fermentation took place at 37 °C and physical maturing at 7 °C. on III group to simulate autumn-winter season cream fermentation took place after physical maturing, at 20 °C. to simulate spring-summer the cream fermentation took place before physical fermentation, at 20 °C. IV group introduced the fermenting cultures in butter kernel. The samples were kept at 9 °C for 3 days for the increase of acid-creating activity.	20.0–37.0	42	>6.0	[24]
Bifidobacterium bifidum ATCC 29521 *Lactobacillus acidophilus* ATCC 4356	6.60–6.66	The cream was pasteurized, cooled to 18–20 °C, and divided in three parts for each replicate. All batches were inoculated with direct vat set culture (freeze-dried) at a level of 15 g/500 L.	18.0–20.0	60	6.57–6.68	[25]
Bifidobacterium lactis B100.6	>9.0	The butters were prepared in seven repetitions by churning the pasteurized cream (30% (v/v) fat) with the probiotic bacteria. The prepared butters were stored at 6 °C for 4 weeks.	–	28	>7.4	[26]

2.3 Continuous Butter Making Process

Most continuous butter production processes are based on the "Fritz method," the German scientist who designed the first equipment for this purpose. The current equipment have some points of difference in relation to the originally built one, but follow its operating principles containing a churning section, separation section, and working sections containing a vacuum chamber (*see* **Note 11**). At the output of the equipment, the butter is packaged.

2.3.1 Churning

The heat-treated cream is transferred to the churning section via a pump. There are studies carried out on the production of butter with probiotics that involve the addition of microencapsulated probiotics to the butter in the churning stage (Table 1). In this step, the cream is churned in a horizontal cylinder with an adjustable speed rotary beater. The phase inversion occurs in a few seconds and the speed of the beater regulates the size of the grains formed, which influences the fat loss to the buttermilk (*see* **Note 12**). Churning temperature is particularly important as phase inversion will only take place if there is enough liquid fat (*see* **Note 13**). In general, the churning temperature is around 10 to 12 °C. The fat content in the buttermilk should not be higher than 0.3 to 0.5% if there is an optimized churning (*see* **Note 14**).

2.3.2 Buttermilk Remotion

After phase separation, the buttermilk and buttermilk mixture are carried from the churning section to the separation section. This section consists of a horizontal, slow-rotating sieve drum with adjustable speed where the butter grains are retained, while the buttermilk passes through a finely meshed wire screen (*see* **Note 15**).

2.3.3 Working

The butter grains, now gathered in larger lumps, are then transferred into the first working section in which a pair of parallel contra-rotating endless screw transports the butter forward and "squeeze" most of the remaining buttermilk out of the product (*see* **Note 16**). This step allows for the water content to be adjusted so that probiotic culture addition and/or salting can be carried out if required without exceeding the limit of 16 g/100 g water in the final product. If the cream used in butter manufacturing has not been inoculated with probiotics, they can also be added at this stage (Table 1). Some butter machines are equipped with a couple of working units consisting of perforated plates interspersed with mixing vanes where the intensive working of the butter is performed. Another approach is to install a medium-shear mixer after the first working section for the same purpose. From the first working section the butter is conveyed either directly or indirectly via a butter pump to the second working section, where the final working takes place (*see* **Note 17**). The working temperature must be kept low (14–16 °C), as this temperature determines the size and the composition of the continuous fat phase, and thereby the extent of the three-dimensional crystal network (*see* **Note 18**).

2.3.4 Salting

Salt can be added in the last part of the first working section by a dosage pump with adjustable capacity and mixed into the butter by the working units. Subsequent working of the butter is accomplished in a short time that is insufficient for dissolving large salt grains (*see* **Note 19**).

3 Notes

1. The buttermilk resulting from the production of butter made with fermented cream is acidic, which restricts the possibilities of application compared to non-fermented one.
2. The temperature of the cooling step can be adjusted to achieve those apropriated to the multiplication of the probiotic or non-lactic ferment that wants to add. Instead, the probiotic can be added as an ingredient (without multiplying in the cream or butter) in the kneading step.
3. Studies such as those by [27] found that butter produced with sweet cream (without fermenting) from milk from cows raised on pasture had a softer texture and different melting properties than that produced from milk cream from stabled cows fed with feed. Milk from cattle on pasture has advantages in terms of increasing the content of polyunsaturated fatty acids such as omega 3, vaccenic acid, and CLA while reducing the levels of omega 6 and palmitic acid [28]. During the summer when cows are on pasture, dairy fat is less saturated and softer than winter cream, which contains higher levels of saturated fatty acids, resulting in harder fat. In general, the rapid cooling of the cream leads to the formation of crystals with low levels of liquid-free fat, which makes the texture of the butter harder and with lower spreadability characteristics. This characteristic can be improved with the method of maturation of the cream where it has its temperature increased in the initial stage and then reduced in two distinct stages. This technique involves cooling the cream to 20 °C, after heat treatment, keeping it for a few hours at this temperature, cooling it to 16 °C, keeping it for 2 to 3 hours, and, finally, cooling it down to the churning temperature. This maturation method leads to the separation of triacylglycerols with a high melting point from those with a low melting point, forming crystals that have laminated structures with the first ones in the center and layers of the second ones that are formed with the decrease in temperature. Butter produced with cream cooled in stages has a higher liquid fat content than that produced with cream cooled quickly and therefore has a softer and more spreadable consistency.

4. There may be differences in the polymorphism of milk fat crystals as a function of the cream's cooling speed [29]. It is noteworthy that the polymorphism is the phenomenon in which milk fat (solid) crystallizes in more than one form, presenting three-dimensional structures of crystalline packaging, giving it a distinct property.
5. Rapid cooling of the cream does not cause changes in the rheological profile and microstructure of the butter. However, slow cooling of aged cream will result in butter with a firm and brittleness texture, due to the formation of a denser crystal network. Butters produced from unripened creams are mainly formed by α- and β'- crystals and by few crystals in the β- form. Maturation results in a transition of crystals from the α- to β'-form and to the β- form which is formed by the recrystallization of fat. This alteration in the structure of the cream facilitates the next stage of obtaining butter, which consists of churning the cream [30]. The chemical composition and the mechanical treatment employed in obtaining butter can also affect the fat crystal network. Fat crystals are present both in the continuous fat phase and within milk fat globules [29].
6. This method favors the formation of crystals with a laminated structure, increases the liquid fat content, and reduces the firmness of the butter compared to the "heat–cool–cool" method. If the objective is to churn sweet cream, a frequently used cooling procedure is to start by chilling at 8 °C for at least 2 hours, regardless of the dairy fat composition. The cream is then heated to the temperature indicated by the composition of the fat considering the iodine index, and the higher this index, the lower the intermediate heating temperature in the cream maturation process.
7. These temperature variations are made to achieve adequate crystallization of the milk fat and to facilitate and/or optimize the separation of the butter grains. Different temperature combinations can be suitable for creams produced in summer or winter.
8. The addition of probiotics has also been widely discussed in terms of food safety since some strains can be used as protective cultures (bioprotection). This characteristic is associated with some species of *Lactobacilli*, *Streptococci*, *Enterococci*, *Lactococci*, and *Bifidobacteria* having a long history of safe use, proven antimicrobial properties, ability to naturally dominate the microflora, and occupy the ecological niche during the storage of products [31].
9. The process and equipment used to manufacture butter can be dependent on many factors, including production volume. For small-scale operations and artisanal production, the butter maker or churn is better adaptable and requires less investment.

In general, 100 L of raw milk yields 4 to 5 kg of butter. There are butter churns of different sizes ranging from those for around 5 kg of cream at 40% fat to those for processing several hundred kilos of cream. The typical mixer consists of a horizontal stainless-steel barrel that rotates around a shaft or a cylindrical tank with a rotating rod or paddle inside.

10. In wet salting, salt is moistened with water before being added to butter as a dough. This method leads to the rapid solubilization of salt in butter. Salting in brine requires the amount of water in the butter to be low. In this stage of working, it is common to use vacuum (20 kPa). After adjusting the moisture content, working continues, but with less vacuum intensity, as excess can lead to the migration of liquid fat and the presence of free oil droplets in the butter.
11. The capacities of these continuous equipment range from 500 to 15,000 kg/h.
12. Both very high and very low speeds will increase fat loss. The general rule is that the lowest speeds should be used and will result in grains with a diameter of 2 to 4 mm. The optimum churning speed depends on the fat content and temperature of the cream. Lower speeds are used for higher fat content and higher temperature.
13. If the liquid fat content is very low, high rotational speeds are needed to increase the temperature of the cream until there is enough liquid fat. However, raising the temperature by mechanical agitation consumes a lot of energy. However, if the churning temperature is too high, there is a loss of fat in the buttermilk and an increase in butter moisture.
14. If the sweet buttermilk (from unfermented cream butter) is to be reused in other dairy products such as cheese or powdered milk, the fat present in it will also be reused.
15. It is especially important to keep the temperature of the butter low through the entire process, and an efficient way to do that is to cool the butter grains in the separation section before they gather into larger lumps. This cooling can be achieved by spraying the butter grains either with chilly water or even better with recirculated cooled buttermilk, which will not cause dilution. Another way of controlling the temperature of the butter grains is circulating chilled water in the jacket of the separating section, but this is not as efficient as spraying the butter grains.
16. Different parameters influence the water content such as low-fat content of the cream, increased churning temperature, inadequate churning speed, size of butter grains, and inadequate working temperature. It should be noted that the interaction between these parameters is very strong, so careful adjustment of their effect must be done.

17. It is important that the working intensity is high enough to ensure a homogeneous texture in the butter. An intensity that is too low will result in loose or free moisture in the product, and an intensity that is too high will result in a greasy and sticky consistency.
18. The working temperature can be controlled by circulating chilled water in the jacket of the two working sections.
19. Undissolved grains will attract moisture during storage, which will result in free water droplets in the butter and reduced keeping quality. It is therefore necessary to use very fine-grained salt (average particle size around 15 mm), which could be added as a suspension (e.g., 100 g salt in 100 g water). It is critical that the salt is not contaminated, especially with copper and iron, as this will reduce dramatically the oxidative stability of the butter.

References

1. O'Callaghan, T. F. (2022). Butter and buttermilk. In: McSweeney, P. L. H., & Mcamara, J. P. *Encyclopedia of dairy sciences* (3rd edition, 5, 1-7). Elsevier. https://doi.org/10.1016/B978-0-12-818766-1.00230-0
2. Parmar P, Lopez-Villalobos N, Tobin JT, Murphy E, Crowley SV, Kelly AL, Shalloo L (2021) Development and evaluation of a processing sector model for butter manufacture using a mass balance technique at two dairy processing sites. Int J Dairy Technol 74(1): 192–201. https://doi.org/10.1111/1471-0307.12737
3. EMBRAPA – Empresa Brasileira de Pesquisa Agropecuária (2020) *ANUÁRIO leite 2020: leite de vacas felizes.* São Paulo: Texto Comunicação Corporativa, 2020. Disponível em: http://www.infoteca.cnptia.embrapa.br/handle/doc/1124722. Acesso em: 10 ago. 2022
4. Astrup A, Rice Bradley B, Thomas Brenna J, Delplanque B, Ferry M, Torres-Gonzalez M (2016) Regular-fat dairy and human health: A synopsis of symposia presented in Europe and North America (2014–2015). Nutrients 8(8): 463. https://doi.org/10.3390/NU8080463
5. Lordan R, Tsoupras A, Mitra B, Zabetakis I (2018) Dairy fats and cardiovascular disease: do we really need to be concerned? Foods 7(3). https://doi.org/10.3390/FOODS7030029
6. Codex Alimentarius. (1971). *Standard for Butter.* CXS 279-1971, FAO. Disponível em: https://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXS%2B279-1971%252FCXS_279e.pdf. Acesso em: 21 dez. 2022
7. USDA - United States Department of Agriculture (1989) United States standards for grades of butter. Agricultural Marketing Service, Dairy Division. Available at: https://www.ams.usda.gov/sites/default/files/media/Butter_Standard%5B1%5D.pd
8. BRASIL 1996 Portaria n° 146, de 07 de março de 1996. Diário Oficial da União, n°. 48, seção 1, de 11 mar. 1996
9. BRASIL 2002 Resolução n° 4, de 28 de junho de 2000. Institui o produto denominado "Manteiga Comum", para comercialização exclusiva no território nacional. Diário Oficial da União, n°. 128, seção 1, de 05 jul. 2000
10. Leroy F, De Vuyst L (2004) Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends Food Sci Technol 15(2):67–78. https://doi.org/10.1016/J.TIFS.2003.09.004
11. Zheng J, Wittouck S, Salvetti E, Franz CMAP, Harris HMB, Mattarelli P, O'toole PW, Pot B, Vandamme P, Walter J, Watanabe K, Wuyts S, Felis GE, Gänzle MG, Lebeer S (2020) A taxonomic note on the genus *lactobacillus*: description of 23 novel genera, emended description of the genus *lactobacillus* beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. Int J Syst Evol Microbiol 70(4): 2782–2858. https://doi.org/10.1099/IJSEM.0.004107
12. Barros, C. P., Verruck, S., Prudêncio, E. S., Zacarchenco, P. B., & Cruz, A. G. (2020).

Probióticos, prebióticos, paraprobióticos e pós-bioticos de nova geração. In: Cruz, A. G., Silva e Alves, A. T., Prudêncio, E. S., Esmerino, E. A., Spadoti, L. M., Silva, M. C., Messora, M. R., Zacarchenco, P. B., & Pimentel, T.C. *Probióticos e Prebióticos - desafios e avanços.* (Cap. 1, p. 24-51). Editora Setembro

13. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC, Sanders ME (2014) Expert consensus document: the international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol 11(8):506–514. https://doi.org/10.1038/nrgastro.2014.66
14. Cuevas-González PF, Liceaga AM, Aguilar-Toalá JE (2020) Postbiotics and paraprobiotics: from concepts to applications. Food Res Int 136:109502. https://doi.org/10.1016/J.FOODRES.2020.109502
15. Barros CP, Guimaraes JT, Esmerino EA, Duarte MCK, Silva MC, Silva R, Ferreira MB, Sant'ana AS, Freitas MQ, Cruz AG (2020) Paraprobiotics and postbiotics: concepts and potential applications in dairy products. Curr Opin Food Sci 32:1–8. https://doi.org/10.1016/J.COFS.2019.12.003
16. Nataraj BH, Ali SA, Behare PV, Yadav H (2020) Postbiotics-parabiotics: the new horizons in microbial biotherapy and functional foods. Microb Cell Factories 19(1). https://doi.org/10.1186/S12934-020-01426-W
17. Bondarchuk OO (2018) Influence of temperature regimes of ripening and fermentation stages on the physical and chemical properties of cream and sour-cream butter quality indicators. Food Sci Technol 12(3):57–63. https://doi.org/10.15673/fst.v12i3.1040
18. Ceylan O, Ozcan T (2020) Effect of the cream cooling temperature and acidification method on the crystallization and textural properties of butter. LWT 132:109806. https://doi.org/10.1016/J.LWT.2020.109806
19. da Silva MN, Tagliapietra BL, Pivetta FP, Richards NSP d S (2022) Nutritional, functional, and sensory profile of added butter from *lactobacillus acidophilus* encapsulated and hyposodium salt. LWT 161:113385. https://doi.org/10.1016/J.LWT.2022.113385
20. Minj S, Anand S (2020) Developing probiotic butter by incorporating encapsulated probiotic organisms. Dairy Sci Public Database 103 (Suppl. 1) https://openprairie.sdstate.edu/dairy_pubdb/2265
21. Minj S, Anand S (2022) Development of a spray-dried conjugated whey protein hydrolysate powder with entrapped probiotics. J Dairy Sci 105(3):2038–2048. https://doi.org/10.3168/jds.2021-20978
22. Ferreira L, Borges A, Gomes D, Dias S, Pereira C, Henriques M (2020) Adding value and innovation in dairy SMEs: from butter to probiotic butter and buttermilk. J Food Process Preserv 46(8):e14867. https://doi.org/10.1111/JFPP.14867
23. Bellinazo PL, Vitola HRS, dos Cruxen CES, Braun CLK, dos Hackbart HCS, da Silva WP, Fiorentini ÂM (2019) Probiotic butter: Viability of Lactobacillus casei strains and bixin antioxidant effect (*Bixa orellana* L.). J Food Process Preserv 43(9):e14088. https://doi.org/10.1111/JFPP.14088
24. Musiy L, Tsisaryk O, Slyvka I, Mykhaylytska O, Gutyj B (2017) Study of keeping probiotic properties of sour-cream butter at storage. EUREKA Life Sci 2(2):27–33. https://doi.org/10.21303/2504-5695.2017.00318
25. Erkaya T, Ürkek B, Doğru Ü, Çetin B, Şengül M (2015) Probiotic butter: stability, free fatty acid composition and some quality parameters during refrigerated storage. Int Dairy J 49:102–110. https://doi.org/10.1016/J.IDAIRYJ.2015.04.011
26. Olszewska M, Staniewski B, Laniewska-Trokenheim L (2012) Cell viability of *Bifidobacterium lactis* strain in long-term storage butter assessed with the plate count and fluorescence techniques. Czech J Food Sci 30(5):421–428. https://doi.org/10.17221/330/2011-CJFS
27. O'Callaghan TF, Faulkner H, McAuliffe S, O'Sullivan MG, Hennessy D, Dillon P, Kilcawley KN, Stanton C, Ross RP (2016) Quality characteristics, chemical composition, and sensory properties of butter from cows on pasture versus indoor feeding systems. J Dairy Sci 99(12) https://doi.org/10.3168/jds.2016-11271:9441
28. Alothman M, Hogan SA, Hennessy D, Dillon P, Kilcawley KN, O'Donovan MA, O'Callaghan TF (2019) The "grass-fed" milk story: understanding the impact of pasture feeding on the composition and quality of bovine milk. Foods 8(8). https://doi.org/10.3390/FOODS8080350
29. Prudencio ES, Amboni RDMC, Fritzen-Freire CB, Verruck S, Esmerino EA, Guimaraes JT, Cappato LP, Silva HLA, Pimentel TC, Chaves ACSD, Cruz AG, Zacarchenco PB (2017) Manteiga e Creme de Leite. In: Cruz AG, Zacarchenco PB, Oliveira CAF, Corassin CH (eds) *Processamento de Produtos Lácteos*, vol 3, 1st edn. Elsevier, pp 115–135

30. Rønholt S, Kirkensgaard JJK, Pedersen TB, Mortensen K, Knudsen JC (2012) Polymorphism, microstructure, and rheology of butter. Effects of cream heat treatment. Food Chem 135(3):1730–1739. https://doi.org/10.1016/j.foodchem.2012.05.087
31. Ois Bourdichon F, Arias E, Babuchowski A, Uckle AB, Dal Bello F, Dubois A, Fontana A, Fritz D, Kemperman R, Laulund S, Mcauliffe O, Miks MH, Papademas P, Patrone V, Sharma DK, Sliwinski E, Stanton C, von Ah U, Yao S, Morelli L (2021) The forgotten role of food cultures. FEMS Microbiol Lett 368(7):73230. https://doi.org/10.1093/femsle/fnab085

Chapter 5

Probiotic Plant-Based Beverages

Tahis R. Baú, Danielle C. B. H. Ferreira, Cintia L. Handa, Fernando S. de Lima, and Tatiana Colombo Pimentel

Abstract

Vegetable matrices are suitable substrates for obtaining probiotic plant-based beverages. The development of these products aims to serve a new segment of consumers who prefer plant-based foods or have restrictions on consuming dairy products. Here we will describe the process of obtaining soy, oat, and rice extracts. Next, we will discuss the fermentation process to obtain probiotic beverages.

Key words Fermentation, Soy, Oat, Rice, Functional food, Plant-based drinks, Milk alternatives

1 Introduction

Demand for probiotic plant-based beverages has grown worldwide among dairy and non-dairy consumers. Consuming probiotic beverages to improve gut health is not new [1]. However, the demand for these products also includes rising vegetarianism and emerging veganism, lactose intolerance, allergenicity for dairy products, dyslipidemia, and consumer demand for differentiated products [2, 3].

Non-dairy plant-based food matrices such as aqueous extracts of plant-based cereals and legumes have successfully been used to produce probiotic beverages. These substrates contain nutrients easily assimilated by probiotics, stimulating the growth of single and mixed cultures during fermentation [3–5] and providing better food product digestibility. The primary sources used to develop probiotic plant-based beverages are soybean, malt, wheat, barley, tree nuts, rice, and oat, which are suitable substrates for microbial growth [3].

Soy extract (commonly known as soymilk) is rich in compounds important for nutrition and beneficial for health [6]. Soy extract has a similar appearance and chemical composition to animal milk and can be used as a substrate for fermentation by lactic acid bacteria [7]. Soy extract has 3.0% protein, 2.0% lipids, and 2.0%

Adriano Gomes da Cruz et al. (eds.), *Probiotic Foods and Beverages: Technologies and Protocols*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-3187-4_5,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2023

carbohydrates, mainly sucrose, raffinose, and stachyose [8]. This product is an alternative to milk due to its protein quality, absence of cholesterol and lactose, and functional properties. However, consumption of this product is limited due to the characteristic flavor and astringent potential of soybeans due to the presence of lipoxygenase enzymes and non-digestible oligosaccharides [9].

Oat (*Avena sativa* L.) is a gluten-free cereal with significant nutritional and functional values, mainly due to its high content of β-glucans. Intake of these soluble fibers has been associated with reduced serum cholesterol and risk of cardiovascular disease [10]. The utilization of this information is authorized by the U.S. Food and Drug Administration (FDA) and European Food and Safety Agency (EFSA) to appear on food labels that contain the minimum amount of β-glucan required for such a health claim. Other oat constituents, such as avenanthramides, tocols, sterols, phytic acid, and avenacosides, have also demonstrated diverse health benefits, including anticarcinogenic and antihyperglycemic activities and improvements in gastrointestinal disorders [11].

Rice is a basic and important food for many of the world's population, and the *Oryza sativa* L. is the most widely grown. The rice grain has a high concentration of carbohydrates, mainly starch, and lower concentrations of vitamins and minerals [12]. The rice grain has potential health benefits so that it can be used as a matrix for plant-based extracts and a vehicle for producing probiotic foods [2]. Plant-based rice extract is a non-dairy beverage extracted from ground rice grain in water followed by homogenization, obtaining emulsions in the water phase of the components present in the rice grain [13–15]. The emulsion formed is a colloidal system constituted by a continuous phase (water) and a dispersed phase (particles). Fraction protein, starch granules, solid parts of rice matrices, and lipid droplets are particles that may be present in the dispersed phase [16, 17]. Plant-based rice extract has been considered an excellent alternative to cow's milk due to health concerns [18–20], is cheaper, and is environmentally friendly [13, 17]. However, plant-based rice extract has different sensory characteristics, stability, and nutritional composition than cow's milk [15].

The aqueous extracts of plant materials, which form the base for probiotic plant-based beverages, are prepared by cleaning, dehulling, soaking, and milling the raw materials in water. Alternatively, it can also be dry milled and subsequently solubilized in water. Then, the slurry is filtered to separate the residues [1, 21]. As a result, these products have been noted for having adequate sensory characteristics, minimum recommended viable probiotic numbers, and a high level of beneficial substances in human nutrition, such as vitamins, minerals, antioxidants, dietary fibers, and natural prebiotic compounds [2, 4, 22]. However, the food matrix is significantly changed during the fermentation

process due to the production of acids, volatile compounds, and other transformations that alter the sensory characteristics of the beverage. Therefore, preparing probiotic plant-based beverages requires they possess desirable sensory, physical, and chemical characteristics and meet probiotic requirements.

Plant-based fermented beverages can be produced through microbial fermentation processes that can occur by the addition of starter cultures (culture-dependent ferments) or naturally (spontaneous ferments) [23]. Industrially produced probiotic products often use starter cultures to ensure product standardization. Industrial microbial cultures include liquid, frozen, freeze-dried, or atomized concentrated microorganisms for starter culture propagation and subsequent inoculation or the use of readily soluble cultures that allow direct vat set (DVS) inoculation [24]. The DVS inoculation of probiotics is convenient for storage and commercial applications. At the same time, freeze-dried concentrated microorganisms are widely used due to their intense fermentation activity and low storage and transportation costs [25, 26].

The probiotic strains used, besides having GRAS (Generally Recognized As Safe) and QPS (Qualified Presumption of Safety) status, recognized by the European Safety Authority—EFSA, must comply with the requirements to be considered probiotic and technological criteria to be employed in beverages, such as: grow in the food matrix, resist the technological processing steps, not produce undesirable sensory compounds, tolerate storage conditions, and resist marketing conditions [24]. Different species of *Lactobacilli* and *Bifidobacteria* have been reported in probiotic plant-based beverages, such as *Limosilactobacillus fermentum*, *Limosilactobacillus reuteri*, *Lactobacillus acidophilus*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus casei*, and *Bifidobacterium* sp. In addition, other studies of mixed plant-based substrates and probiotic strains are being developed to produce beverages of particular flavor characteristics [3]. This chapter describes the processes for obtaining soy (*see* Subheading 2.1), oat (*see* Subheading 2.2), and rice extract (*see* Subheading 2.3). After obtaining one of the extracts, the fermentation procedure can be carried out according to Subheading 3.

2 Plant-Based Aqueous Extract

The processes for obtaining soy (*see* Subheading 2.1), oat (*see* Subheading 2.2), and rice extracts (*see* Subheading 2.3) will be described.

2.1 Soymilk

2.1.1 Materials

1. Food-grade whole soybeans, full-fat soy flakes, or full-fat soybean flour can be used (*see* **Note 1**).
2. Drinking water.
3. Container or tank for soaking soybean.
4. Stainless steel semi-industrial blender or grinder.
5. Filtration system.
6. System for heat treatment.

2.1.2 Methods

The soy extract can be obtained from the following steps:

1. Selection and removal of major and minor dirt from soybeans through sieving (*see* **Note 2**).
2. Soak soybeans in drinking water at a ratio of 1:3 soy: water, for 3 to 15 h, depending on conditions (*see* **Note 3**).
3. Grind the wet soybeans in a stone mill or hammer mill with additional fresh water. The ratio of water to soybeans is usually 6:1 to 10:1 (*see* **Note 4**).
4. Filter the slurry to separate the soybean extract from the insoluble fiber (okara) through a sieve, cloth, or pressing bag, with or without a wooden press. The slurry also can be filtered on one or more scraped filters in batch or semicontinuous mode or by continuous centrifugation filtration. The okara is usually washed once or twice with cold or hot water, stirred, and re-pressed to maximize soymilk yield. The total volume of the combined filtrate (raw soymilk) is about 6–10 times the original soybean volume.
5. Carry out the heat treatment followed by rapid cooling of the soymilk before fermentation. Heat treatment can be carried out by pasteurization or sterilization using ultra-high temperature (UHT). At the pasteurization, soybean extract is boiled for 10 min (95–98 °C) from the start of boiling, continuously stirring. The processing by UHT of soymilk is usually done by using UHT directly with steam injection, followed by a short holding time of about 5 s at about 145 °C, followed by flashing to remove unpleasant odors and excess steam.

2.1.3 Notes

1. For a better result, you should preferably use clear hilum soybeans to produce soy extract with whiter color, higher yield, and better overall quality.
2. At selection, discard the broken soybeans because the enzymatic reactions that cause the off flavor have already taken place. After the selection, optionally, the soybean can be dehulled to improve the flavor of the soy extract by removing bitter and astringent compounds from the husk.

3. Before soaking, the beans should be rinsed under running water, careful not to damage them. After soaking, the water can be removed and replaced with fresh process water. Soaking the soybeans in cold water causes little or no lipoxygenase activity. However, it requires more time than soaking in warm or hot water. Soaking in hot water has the advantage of removing all adverse enzyme reactions very quickly. Therefore, many processes include a hot blanching step for 15 to 20 min in 3–5 volumes of water at 85 to 90 °C. In addition, soaking in bicarbonate solution plays an essential role in softening the structure of soybeans, which will later create a finer slurry. As a result, soy extraction yield is higher because a large portion of the soybeans can seep through the filter cloth, resulting in higher protein content.
4. If heating has not yet taken place before grinding, hot water is used, or steam is injected to increase the temperature of the soybean slurry as quickly as possible. In addition to inactivating the enzyme, increasing the cooking temperature of soybeans will reduce the viscosity of the oil in soy extract and make it easier for the oil cells to break down. This phenomenon allows oil to be released and further increases the crude fat content of soy extract.

2.2 Oat Extract

2.2.1 Materials

1. Whole meal, hulled, or flakes oats.
2. Drinking water.
3. Container or tank for soaking oat.
4. Stainless steel semi-industrial blender or grinder.
5. Water bath.
6. Optional application of thermostable endo-acting enzymes: α-amylase (120 KNU-T/g) and β-glucanase (1 U/mg), both food grades.
7. Filtration system with mesh opening size up to 20 μm.

2.2.2 Methods

The preparation of oat extract, popularly known as oat “milk,” can be obtained from the following steps:

6. Select and wash whole or hulled oats to remove dirt and unwanted material.
7. Soak whole meal, hulled, or flakes oats in drinking water at a ratio of 4 to 8% (w/v) at room temperature for 12 h (*see* **Note 1**).
8. Grind the oat with their soaking water in a stainless-steel blender or grinder for up to 5 min (*see* **Note 2**). Food industries generally do not perform the soaking step due to the long time required. In this case, the oat can be ground with hot

water at 80–90 °C for up to 5 min to adequately extract proteins, β-glucan, vitamins, minerals, etc.

9. If the objective is to reduce beverage viscosity and/or increase protein extraction, add 0.15% α-amylase (w/w; enzyme/oat) and 0.04% β-glucanase (w/w; enzyme/oat) to the slurry and keep it in a water bath at 80 °C for 2 h under moderate agitation (*see* **Note 3**).
10. Cool the slurry to 30 °C and then filter or centrifuge it to pass through a mesh opening up to 20 μm (*see* **Note 4**). The material retained in the mesh can be applied for the elaboration of bakery products.
11. This oat extract must be pasteurized in a water bath, discontinuous or continuous heat exchanger, before inoculating the probiotic culture (*see* Subheading 3).

2.2.3 Notes

1. The ratio of 4 to 8% (w/v; oat/water) is suitable for extracting a significant amount of β-glucan and making the beverage prebiotic. However, oat extract with high amounts of this fiber can show undesirable sensory properties to consumers due to its high viscosity. Therefore, carrying out previous tests with consumers is important to assess the drink's acceptability.
2. High grinding time contributes to greater β-glucan extraction, but its impact on beverage viscosity must be investigated.
3. Termamyl® (Novozymes) is an endo-acting α-amylase used to liquefy oat starch and produces oat-based drinks with moderate viscosity. Beerzym BGHK4® (Erbslöh Geisenheim GmbH) is a β-glucanase that also reduces the viscosity of the beverage through the hydrolysis of β-glucan.
4. High pressure during filtration can make it easier for the slurry compounds to pass through the mesh, especially β-glucan and proteins.

2.3 Rice Extract

2.3.1 Materials

1. Rice grain (fully milled or partially milled), milled broken rice, or rice flour.
2. Drinking water.
3. Container or tank for soaking rice.
4. Stainless steel semi-industrial blender or grinder.
5. Enzymes: α-amylase and glucoamylase (Glucozyme).
6. Filtration system.

2.3.2 Methods

The process of extracting the rice extract ("rice milk") follows the following steps:

1. Prepare the grains, passing them through cleaning, selection, classification, and washing steps. The rice grains can be fully or partially milled (*see* **Note 1**).
2. Hydrate the grains in water in the proportion of 1:2 (w/v) at 2 °C for 5 h.
3. Grind the rice with the hydration water using the stainless-steel blender or grinder for about 3 min until a homogeneous mixture is obtained. Then, for liquefaction, add 0.1% enzyme α-amylase at 90 °C for 30 min in the homogeneous mixture and use 0.1% glucoamylase (Glucozyme) at 55 °C for 12 h to reduce the beverage's viscosity and convert rice extract's starch to simple sugars for consumption in the fermentation process of plant-based rice extract. Saccharification can also be carried out simultaneously with the fermentation step, when applicable, by adding 0.1% glucoamylase (Glucozyme), a fermenting microorganism, to 200 g of rice extract (*see* **Note 2**).
4. Remove coarse particles by filtration, decanting, or centrifugation. These larger or coarse particles can be used in other food products. Next, extract the soluble phase ("rice milk" or rice extract).
5. Add other necessary ingredients to improve the chemical and sensory characteristics of the beverage (*see* **Note 3**).
6. Pasteurize the rice extract before inoculation of the probiotic culture. Pasteurization can occur in a water bath, discontinuous, or continuous heat exchanger.

2.3.3 Notes

1. The process of rice extract involves grain preparation, hydration, and breakdown (size reduction) of grain extracted in water, treated with enzymes to partially break down the starch and facilitate a suspension mixture, filtered, and thermic treatment. Fully milled indicates that the husk, germ, and bran have been removed, and only the endosperm (white rice) remains, while the partially milled only husk has been removed. Rice flour can also be used as a raw material. Fully milled grain may result in better texture in the slurry (paste), but it has high starch content and low content of nutrients, fiber, vitamins, and bioactive components. Several rice grains can be used in plant-based rice processing, but polished rice is the most used raw material. Brown rice is also sometimes used. When rice flour is used to extract the beverage, there will be no need for a milling step, but an effective mixing solution is recommended to create a uniform slurry.
2. Cereals and pseudocereals have a high proportion of starch and form a thick paste when heated above the gelatinization temperature (55–65 °C). Therefore, to prevent and avoid problems

in the later stages of processing, the use of the α-amylase enzyme is required.

3. After obtaining the desired viscosity (thickness), other ingredients can be added [13, 14]. Protein, calcium, and vitamins are examples of essential nutrients required in the rice extract once they are limiting nutrients [13, 27].

3 Fermentation

The general process of obtaining probiotic plant-based beverages using DVS culture will be described. However, depending on the culture used and the substrate, it will probably be necessary to adapt the inoculation and fermentation conditions. In some cases, it is necessary to reactivate the culture, which can be purchased in liquid, freeze-dried, or concentrate-frozen form, before inoculation, so follow the manufacturer's instructions.

3.1 Materials

1. The aqueous extract was obtained according to Subheading 2.
2. Microbial culture: add 2% to 3% of the microbial culture or use the manufacturer's recommendations (*see* **Note 1**).
3. Optional ingredients: Sucrose (6 to12%), thickeners (0.5%), flavorings (0.1%), and other optional ingredients such as pulps, fruits, and prebiotics are recommended.
4. Flasks and incubator oven or industrial fermentation tank.
5. Cooling heating system for beverages.

3.2 Methods

The preparation of probiotic plant-based beverages can be obtained from the following steps:

1. Add the non-volatile or thermolabile ingredients and homogenize for solubilization (aqueous extract, sucrose, thickeners, and others) (*see* **Note 2**).
2. Heat treat the mixture of aqueous extract and other ingredients before receiving the inoculum for fermentation (*see* **Note 3**). It is suggested to use 95 °C for at least 5 min (*see* **Note 4**).
3. Cool the aqueous extract rapidly to fermentation temperature (usually between 25 °C and 43 °C), according to the manufacturer's recommendations (*see* **Note 5**).
4. Add the inoculum, homogenizing with the help of sanitized or sterile utensils.
5. Ferment at the temperature indicated for the microorganism (usually between 25 °C and 43 °C) until the desired final pH (*see* **Note 6**).

6. Cool to 5 °C for 12 h for stabilization and then homogenize, preferably without incorporating air (*see* **Note 7**).
7. Add the flavorings and other thermolabile ingredients.
8. Aseptically fill in appropriate packaging and, preferably, store under refrigeration.

3.3 Notes

1. Check the manufacturer's instructions for use in volumes lower than the recommended in the microbial culture envelope. Generally, for mixed culture envelopes, you should sterilize 1 L of the fermentation substrate at 121 °C for 15 min. After cooling down (~10 °C), add the mixed culture (1 envelope of 5 UC for 1 L of the substrate), and homogenize with sterile utensils. Then distribute into sterile 10 mL containers and freeze quickly at −18 °C until use. In this example, each container will contain enough microbial culture to be used in 5 L of beverage. However, other proportions can be used according to the production scale.
2. Flavoring ingredients or ingredients with thermal instability can be added aseptically after heat treatment. The sanitary quality of these ingredients must be checked beforehand or ensured by the manufacturer through technical reports. Sucrose is usually added to improve flavor and consistency and is used as a fermentation substrate by some starter cultures. In addition, thickeners, fruit pulp or juice, and other compounds can improve the product's stability and acceptability.
3. The aqueous extract must have a typical taste and aroma, an absence of spoiling microorganisms, pathogens, and an absence of fermentation inhibiting substances. In addition, the heat treatment must be carried out in such a way as to guarantee the safety of the product.
4. If the heating is done in an open tank, at a lower temperature and longer time, an increase in the solids content of the aqueous extract will occur. The same effect can be observed with heating at 95 °C for 5 min in a plate heat exchanger under a partial vacuum, where part of the water is evaporated.
5. Heating and cooling operations can be performed on a plate heat exchanger.
6. Generally, aqueous extracts are fermented to a pH of about 4.3. The fermentation time depends on the characteristics of the culture and temperature employed and typically ranges from 4 to 30 h. Some probiotic microorganisms, such as *Bifidobacteria*, can accumulate compounds undesirable for the product during fermentation, such as acetic acid. Therefore, in some cases, the probiotic may not participate in the fermentation

process. In this case, the highly concentrated culture is added at the end of the process, followed by cooling and packaging.

7. Most probiotic microorganisms are anaerobic, and mixing can lead to the incorporation of oxygen into the beverage, reducing the probiotics' viability.

References

1. Udayarajan CT, Mohan K, Nisha P (2022) Tree nuts: treasure mine for prebiotic and probiotic dairy free vegan products. Trends Food Sci Technol 124:208–218
2. Rasika DM, Vidanarachchi JK, Rocha RS et al (2021) Plant-based milk substitutes as emerging probiotic carriers. Curr Opin Food Sci 38:8–20
3. Salmerón I (2017) Fermented cereal beverages: from probiotic, prebiotic and synbiotic towards nanoscience designed healthy drinks. Lett Appl Microbiol 65(2):114–124
4. Martins EMF, Ramos AM, Vanzela ESL et al (2013) Products of vegetable origin: a new alternative for the consumption of probiotic bacteria. Food Res Int 51(2):764–770
5. Rathore S, Salmerón I, Pandiella SS (2012) Production of potentially probiotic beverages using single and mixed cereal substrates fermented with lactic acid bacteria cultures. Food Microbiol 30:239–244
6. Arumugam S, Dioletis E, Paiva R et al (2020) Fermented soy beverage Q-CAN plus consumption improves serum cholesterol and cytokines. J Med Food 23(5):560–563
7. Baú TR, Garcia S, Ida EI (2013) Optimization of a fermented soy product formulation with a kefir culture and fiber using a simplex-centroid mixture design. Int J Food Sci Nutr 64(8): 929–935
8. Peng X, Wang Y, Xing J et al (2017) Characterization of particles in soymilks prepared by blanching soybeans and traditional method: a comparative study focusing on lipid-protein interaction. Food Hydrocoll 63:1–7
9. Šertović E, Sarić Z, Barać M et al (2019) Physical, chemical, microbiological and sensory characteristics of a probiotic beverage produced from different mixtures of cow's milk and soy beverage by lactobacillus acidophilus La5 and yoghurt culture. Food Technol Biotechnol 57(4):461–471
10. Martínez-Villaluenga C, Peñas E (2017) Health benefits of oat: current evidence and molecular mechanisms. Curr Opin Food Sci 14:26–31
11. Angelov A, Yaneva-Marinova T, Gotcheva V (2018) Oats as a matrix of choice for developing fermented functional beverages. J Food Sci Technol 55:2351–2360
12. Muthayya S, Sugimoto JD, Montgomery S, Maberly GF (2014) An overview of global rice production, supply, trade, and consumption. Ann N Y Acad Sci 1324:7–14
13. Sethi S, Tyagi SK, Anurag RK (2016) Plant-based milk alternatives an emerging segment of functional beverages: a review. J Food Sci Technol 53(9):3408–3423
14. Lamothe M, Rivero-Mendoza D, Dahl W (2020) Plant-based milks: Rice. EDIS FSHN20 50:1–4
15. Silva ARA, Silva MMN, Ribeiro BD (2020) Health issues and technological aspects of plant-based alternative milk. Food Res Int 131:1–17
16. Briviba K, Gräf V, Walz E et al (2016) Ultra high pressure homogenization of almond milk: Physico-chemical and physiological effects. Food Chem 192:82–89
17. Bocker R, Silva EK (2022) Innovative technologies for manufacturing plant-based non-dairy alternative milk and their impact on nutritional, sensory and safety aspects. Future Foods 5:1–12
18. Faccin GL, Miotto LA, do Nascimento Vieira L et al (2009) Chemical, sensorial and rheological properties of a new organic Rice bran beverage. Rice Sci 16(3):226–234
19. Van Winckel M, Velde SV, De Bruyne R, Van Biervliet S (2011) Clinical practice: vegetarian infant and child nutrition. Eur J Pediatr 170(12):1489–1494
20. Biswas S, Sircar D, Mitra A, De B (2011) Phenolic constituents and antioxidant properties of some varieties of Indian rice. Nutr Food Sci 41(2):123–135
21. Mäkinen OE, Wanhalinna V, Zannini E, Arendt EK (2016) Foods for special dietary needs: non-dairy plant-based Milk substitutes and fermented dairy-type products. Crit Rev Food Sci Nutr 56(3):339–349

22. Panghal A, Janghu S, Virkar K et al (2018) Potential non-dairy probiotic products – a healthy approach. Food Biosci 21:80–89
23. Dimidi E, Cox SR, Rossi M, Whelan K (2019) Fermented foods: definitions and characteristics, impact on the gut microbiota and effects on gastrointestinal health and disease. Nutrients 11(8):3–26
24. Martin JGP, Lindner JD (eds) (2022) Microbiologia de alimentos fermentados. Bluncher, São Paulo
25. Hu D, Wu J, Jin L et al (2021) Evaluation of Pediococcus pentosaceus strains as probiotic adjunct cultures for soybean milk post-fermentation. Food Res Int 148:110570
26. Peiren J, Buyse J, De Vos P et al (2015) Improving survival and storage stability of bacteria recalcitrant to freeze-drying: a coordinated study by European culture collections. Appl Microbiol Biotechnol 99(8):3559–3571
27. Vanga SK, RaghavanV (2018) How well do plant based alternatives fare nutritionally compared to cow's milk? J Food Sci Technol 55:10–20

Chapter 6

Probiotic Plant-Based Cheese

Tahis R. Baú, Danielle C. B. H. Ferreira, Cintia L. Handa, Fernando S. de Lima, and Tatiana Colombo Pimentel

Abstract

Due to several factors, the demand for plant-based cheese has increased. However, formulating products with characteristics similar to cheese made with animal milk is still a challenge for researchers and industries. Here we will describe the process of obtaining probiotic pea cheese, probiotic tofu, probiotic soy-based cream cheese, and probiotic chickpea petit suisse cheese.

Key words Pea cheese probiotic, Probiotic tofu, Chickpea probiotic petit suisse cheese, Functional food

1 Introduction

Probiotic plant-based cheeses are products made from vegetable sources, usually legumes, including non-dairy fats or proteins, which result in a cheese analogous to that made with animal milk. It has benefits for the health of the consumer. Ethical reasons, sustainability, animal welfare, and health, such as lactose intolerance, milk allergy, and cholesterol issues, are the main consumer concerns that drive interest in plant-based dairy alternatives [1, 2]. The increased production and demand for plant-based cheese alternatives are also due to the increasing number of people following vegan diets [3].

The cheeses of vegetable origin differ sensorially from the cheeses of animal origin due to their composition. The plant-based cheese does not present the physical and sensory characteristics of dairy-based cheese, whereas the comparisons between flavor, taste, aroma, mouthfeel, and meltability limit consumer acceptability [2]. In cheese made with animal milk, casein-casein interactions promote stretch and flow functionality, which provides structure to the cheese matrix. In plant-based cheese, the formation of compact gel networks does not occur in the same way as with

Adriano Gomes da Cruz et al. (eds.), *Probiotic Foods and Beverages: Technologies and Protocols*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-3187-4_6,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2023

casein. As rennet does not induce coagulation in plant milk, other protein coagulation methods should be employed [4–6]. Therefore, applying enzymes, acids, lactic acid fermentation, or heat treatments are technologies that need to be considered [7]. This is because plant proteins have larger molecule sizes and more complex quaternary structures than milk proteins, in addition to having properties such as cross-linking and hydrophobicity [8]. Thus, these products are formed by lipids embedded in polysaccharides or protein matrices, forming a colloidal dispersion [6].

Many types of plant-based cheeses are formulated using combinations of oils (e.g., coconut or palm oil) and starches (e.g., potato or tapioca starch) [1]. The main ingredients for the formulation of a plant-based cheese are water, lipids, and vegetable proteins, which can be included as stabilizers, emulsifying salts, acidifying agents, preservatives, and flavors [9, 10]. In addition, a combination of ingredients has been used to provide a product with structure, viscosity, and melting similar to traditional cheese [5]. Although most of the time, a combination of ingredients is necessary, the primary raw materials used in producing plant-based cheeses are soybeans, peas, cashew nuts, coconut oil, oats, almonds, palm fruit oil, and corn zein [3, 11].

Peas represent a good alternative to produce plant-based cheeses as they have a low production cost and high protein content, which also stand out for their excellent gel formation. The gel is mainly formed by the presence of globulins, such as legumin and vicilins, representing 70–80% of the total protein content [11, 12]. In addition, pea cheese proved to be a suitable substrate for fermentation, and protein gels can be produced with 10% protein content and 10% olive oil levels without compromising gel hardness [11].

Soybean is a food cholesterol-free, low in sodium, a good source of nutrients, and a suitable medium for probiotic growth [13, 14]. Tofu is a plant-based cheese made from soy milk, one of the most important and popular foods in Asian countries, and is widely accepted worldwide. It is gaining popularity in Western countries due to its health benefits [15]. The fermentation increases the nutritional value and helps remove soy's taste, which many consumers do not accept [16]. Probiotic tofu can be a healthy alternative for vegans and vegetarians while positively affecting consumers' health and improving the taste of soy cheese.

Soy-based cream cheese is a cream cheese analog known for its creaminess and spreadability. Chemically, it can be defined as a microgel with a structure formed of protein-covered soy fat globules [4, 17], resulting from the homogenization of tofu, fat, and stabilizers. Therefore, its processing begins with the acidic coagulation of soymilk to obtain tofu. However, there are still few studies [18–20], on this cream cheese analog, particularly with the addition of probiotics. Nevertheless, advances in processing, such as

membrane technology, enzymatic protein modification, and high hydrostatic pressure and ultrasound treatments, are promising to contribute to this product's sensory characteristics, texture, and cost efficiency [21].

The fermentation of these substrates to obtain a probiotic plant-based cheese is an alternative that can improve the products' nutritional, sensory, and shelf life since starter cultures can be selected that, in addition to promoting acidification, can produce extracellular polysaccharides that collaborate with the firmness of the product. This chapter describes the processes for obtaining probiotic pea cheese (*see* Subheading 2), probiotic tofu (*see* Subheading 3), probiotic soy-based cream cheese (*see* Subheading 4), and probiotic chickpea petit suisse cheese (*see* Subheading 5).

2 Pea Cheese Probiotic

Pea cheese is plant-based, produced by solubilizing pea protein in water and blending it with oil to create a plant-based emulsion. Coagulation of pea protein for gel formation can occur through the action of heat, high pressure, acidification, and fermentation. There is still a little exploration of fermentation-induced pea gel formation in this last one. Concerning the production of probiotic pea cheese, there are still few studies that explore this process. The primary materials and processing steps for the pea cheese probiotic are described below:

2.1 Preparation of Probiotic Pea Cheese

2.1.1 Materials

1. Pea protein isolate (PPI).
2. Extra-virgin olive oil.
3. Sucrose.
4. Glucose.
5. Salt.
6. Starter culture (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*).
7. Probiotic culture (e.g., *Lactobacillus acidophilus, Lacticaseibacillus paracasei,* and *Bifidobacterium*).

2.1.2 Methods

1. Suspend a pea protein isolate (PPI) in distilled water until a final protein concentration of 10% (w/w) (*see* **Note 1**).
2. Stir the mixture at 9500 rpm for 3 min with Ultra-Turrax, to ensure protein hydration.
3. Add 1% glucose (w/w) and 1% (w/w) sucrose during the stirring step to ensure that there is a substrate for the bacteria to grow and acidify the matrix, given the low sugar content of the PPI.

4. Emulsify the mixture using extra-virgin olive oil (around 10% olive oil, w/w) with Ultra-Turrax at 13,500 rpm for 3 min (*see* **Note 2**).
5. Homogenize the sample under continuous operation and high pressure at two stages (150 and 50 bars) in one pass to decrease oil droplet size and avoid phase separation (*see* **Note 3**).
6. Pasteurize (*see* **Note 4**) the matrix at 95 °C for 5 min in a water bath and cool down to 43 °C before microbial inoculation. For starter culture inoculation utilize 0.02% inoculum. Add probiotic culture and mixture. Containers used for preparation must be previously dry autoclaved at 121 °C for 20 min.
7. Ferment at 43 °C until reaching pH 4.5 (*see* **Note 5**).
9. Mold the product.
10. Store at refrigeration temperature.

2.1.3 Notes

1. It is known that 10% protein is an optimal protein concentration for a stable liquid matrix prepared with the PPI. This concentration keeps the matrix density low, which allows easy pre-processing and homogeneous inoculation in a liquid media.

   The use of PPI is a functional starting raw material for fermentation-induced pea protein gels.

   In the case of using another source of raw material for the production of probiotic pea cheese, such as pea protein concentrate (78% protein), it is recommended to adjust the pH of the suspension, as different pH values during pre-treatment of pea protein led to different ratios between soluble and insoluble protein aggregates in the protein slurry before fermentation. Therefore, these are determining factors during the formation of the gel, directly influencing the rheological properties. For example, the solubility of pea protein concentrate is highest at pH 8.0 and lowest at pH 6.0.
2. Olive oil or other oil types provide the matrix with the fat needed for the cheese and help to stabilize the pea proteins in suspension, which will likely sediment over time if only suspended in water. It is recommended that the initial pH of the PPI emulsions be 7.0. In pea protein emulsions, this protein acts as a surfactant due to its excellent emulsifying properties, which contribute to the stability of the emulsion. Thus, avoiding the need to use surfactants.
3. Hydration and homogenization of proteins with two pressure stages are important operations in producing a PPI matrix that guarantees stability and avoids phase separation during fermentation.

4. Pasteurization of the matrix before fermentation is necessary to ensure the safety and growth exclusivity of the inoculated starter culture. Matrices with high starch contents will form a gel before fermentation, and it will be impossible to inoculate bacteria into a liquid medium. Therefore, less purified pea protein ingredients can present challenges during matrix processing. Thus, it is necessary to use purer protein fractions, such as protein isolates (80–95% protein).
5. The time required to reach pH 4.5 can vary between 5.5 and 7 h. The final pH affects rheology and meltability. Higher pH values (6.0–7.0) provide the cheese analog with more viscous properties and lower pH (4.5–5.5) with higher elasticity.

3 Probiotic Tofu

Tofu is a soy-based product that is precipitated with coagulants in the form of curds. The curds' size and the pressing time length determine the style of tofu, which can be soft, regular, firm, or extra firm. The probiotic culture can be added before or after the coagulant. However, there are still few studies that have investigated the incorporation of probiotics in tofu. The primary materials and processing steps are described below:

3.1 Preparation of Probiotic Tofu

3.1.1 Materials

1. Food-grade whole soybeans (*see* **Note 1**), full-fat soy flakes, or full-fat soybean flour.
2. Drinking water.
3. Coagulant (*see* **Note 2**).
4. Probiotic cultures.
5. Container or tank for soaking soybean.
6. Stainless steel semi-industrial blender or grinder.
7. Filtration system.
8. System for heat treatment.
9. Incubator for fermentation.

3.1.2 Methods

1. Wash dry whole soybeans and soak them in water overnight. The volume of water is usually 2 to 3 times the volume of the bean.
2. Drain the soaked beans and rinse with fresh water 2–3 times.
3. Ground the wet, clean soybeans in a mill with fresh water. The water:bean ratio is usually in the range of 6:1 to 10:1.
4. Filter the soy milk through a sieve, cloth, or press bag. Remove residue and wash once or twice with water (cold or hot), stirring and pressing again to maximize milk production.

5. Heat raw milk at a boiling temperature and keep it at this temperature for 5–10 min (*see* **Note 3**).
6. Once the milk is heated, transfer it to another container. At the same time, mix the powdered coagulant with a small amount of hot water to make a coagulant suspension. Then add the coagulant to the hot soy milk at 70–85 °C; depending on the type of coagulant used, let the mixture sit for about 10 min so that the proteins can coagulate (*see* **Note 4**).
7. After noting the beginning of coagulation, add the probiotic culture and mix.
8. Transfer the mixture to a sterile press cloth and place it into the mold. Discharge excess liquid by pressing (*see* **Note 5**).
9. Ferment at the temperature indicated for the microorganism (usually 37 °C) until the desired final pH, for about 20 h.
10. Store at refrigeration temperature.

3.1.3 Notes

1. Preferably beans with large seed sizes and light hilum to produce tofu of whiter color, higher yield, and better overall quality.
2. Coagulants can be enzymes, salts, or acids. The widely used ones for tofu making are calcium sulfate, magnesium chloride, or a mixture of both, nigari, and glucono-6-lactone (GDL, known as lactone).
3. To avoid burning the milk at the bottom of the cooking vessel, slow heating with frequent stirring is necessary. Alternatively, soy slurry may be heated before filtering into soymilk. Heating soy milk is important to denature the proteins so that they coagulate into curds in the presence of the coagulant. However, prolonged heat treatment should be avoided as it can destroy nutrients such as essential amino acids and vitamins, Maillard browning, and the development of cooked flavors, leading to lower yields and poor-quality tofu.
4. Factors affecting coagulation include the temperature at which a coagulant is added, the type and concentration of coagulants, the mode of adding coagulants, and the duration of coagulation [14]:
   - The suitable amount of coagulant: coagulates all soy milk and generates a clear whey with an amber or pale yellow color and sweet taste.
   - Excessive coagulant: the whey has a slightly bitter taste and a yellowish color, and the curd has a coarse, hard texture.
   - Insufficient coagulant: the resulting whey is cloudy, with some remains of uncoagulated soy milk.

- High temperature: rapid coagulation resulting in tofu with low water-holding capacity, hard and coarse texture, and thus a low bulk yield.
- Temperature too low: coagulation becomes incomplete, making tofu too soft to retain its shape.
- Time too short: coagulation is incomplete.
- Time too long: the system's temperature decreases to such an extent that the subsequent shaping step becomes complex. For silken tofu, the dwell time should be about 30 min; for regular tofu, 20–25 min; and for firm tofu, 10–15 min.

  The suggested additional temperature and concentration for the main coagulants are: calcium sulfate ($CaSO_4$) 0.5% (w:v) at 80–85 °C, magnesium chloride ($MgCl_2$) 5% (w:v) at 70–72 °C; Glucono-δ-lactone (GDL) 0.020 mM at 80 °C. The incubation time for coagulation is 10 min.

5. The curd is pressed to form tofu. The size of the curd and the length of the pressing time determine the style of tofu produced, which can be soft, regular, firm, or extra firm tofu—the softer the tofu, the lower the protein and fat levels and the higher the water content.

4 Soy-Based Cream Cheese Probiotic

This product is mainly obtained from tofu, which results from the acidic coagulation of soymilk. However, there are still few studies that have investigated the incorporation of probiotics in soy-based cream cheese. The main materials and processing steps are described below:

4.1 Preparation of Probiotic Soy Cream Cheese

4.1.1 Materials

1. Soft or firm tofu (*see* Subheading 3).
2. Palm oil.
3. Carrageenan gum.
4. Pectin.
5. Maltodextrin.
6. Salt.
7. Stainless steel semi-industrial blender or high-pressure homogenizer.
8. Probiotic cultures.

4.1.2 Methods

1. Homogenize (*see* **Note 1**) the tofu with palm oil (5–10%, w/w), carrageenan gum (4%, w/w), pectin (1%, w/w), maltodextrin (2–4%, w/w), and salt (1–3%, w/w). These ingredients

are responsible for the stability and texture of probiotic soy cream cheese (*see* **Note 2**).

2. Add the probiotic culture (*see* **Note 3**) to the soy cream cheese using sanitized or sterilized utensils.
3. Aseptically fill in appropriate packaging and store under refrigeration.

4.1.3 Notes

1. Homogenization time in stainless steel semi-industrial blenders varies from 2 to 5 min to produce a homogeneous emulsion. On an industrial scale, the mixture is homogenized in a high-pressure homogenizer with pressure ranging from 10 to 25 MPa. It is noteworthy that high shear rates make formulations more spreadable.
2. In addition to maltodextrin, soy protein concentrate or isolate can be added to the mixture to obtain a smooth soy cream cheese. Carrageenan gum and pectin increase the viscoelasticity of the cream and reduce product syneresis during cold storage. Furthermore, salt concentrations above 6 g/kg and high-fat content (>280 g/kg) can also reduce syneresis [17].
3. It is recommended that the probiotic concentration be at least 8 log CFU/g because there is a reduction in this value during storage and after ingestion of the product. Therefore, viability tests of probiotics are necessary to ensure a concentration above 6 log CFU/g of product at the time of consumption and in the distal part of the gastrointestinal tract.

5 Chickpea Probiotic Petit Suisse Cheese

Chickpea probiotic petit suisse cheese is the food produced from the fermentation of chickpea extract, followed by obtaining quark cheese. This product can be flavored and added with emulsifiers and stabilizers. There are still few studies that investigate the use of chickpeas for the preparation of petit suisse. The main materials and processing steps are described below:

5.1 Preparation of Chickpea Probiotic Petit Suisse Cheese

5.1.1 Materials

1. Food-grade whole chickpea.
2. Drinking water.
3. Container or tank for soaking chickpeas.
4. Stainless steel semi-industrial blender or grinder.
5. Water bath.
6. Probiotic cultures (*see* **Notes 1** and **2**).
7. Stainless steel semi-industrial blender or grinder.
8. Filtration system.

9. System for heat treatment.
10. Incubator for fermentation.

5.1.2 Methods

The preparation of chickpea probiotic petit suisse cheese starts with the preparation of the chickpea extract, and can be obtained by the following steps:

1. Select and wash whole or hulled chickpeas to remove dirt and unwanted material.
2. Soak the chickpea in drinking water at a ratio of 1:3 chickpea: water (w/v) at room temperature for 12 h.
3. Drain the soaked chickpea and rinse with fresh water 2–3 times.
4. Grind the chickpea with water for up to 10 min. The water: chickpea ratio is usually in the range of 6:1 (w/v). Food industries generally do not perform the soaking step due to the long time required. In this case, chickpeas can be ground with hot water at 80–90 °C for up to 5–10 min for adequate extraction of compounds.
5. Cool the slurry to 30 °C and filter through a sieve, cloth, or pressing bag. The slurry also can be filtered on one or more scraped filters in batch or semicontinuous mode or by continuous centrifugation filtration.
6. Add sugar (5–15%, w/w) to the chickpea extract and give heat treatment. Before inoculating the probiotic culture, this chickpea extract must be pasteurized in a water bath, discontinuous or continuous heat exchanger (*see* **Note 3**).
7. Cool the aqueous extract rapidly to fermentation temperature (usually between 37 °C and 43 °C), according to the manufacturer's recommendations.
8. Add the inoculum, homogenizing with the help of sanitized or sterile utensils.
9. Ferment at the temperature indicated for the microorganism (usually between 37 °C and 43 °C) until the desired final pH (*see* **Note 4**).
10. Desorb for up to 12 h at 4–8 °C in a synthetic filter or sieve to get quark cheese (*see* **Note 5**).
11. Add guar gum (0.3–0.7%, w/w) and xanthan gum (0.3–0.7%, w/w), and mix with quark mass (*see* **Note 6**).
12. Add, if you prefer, fruit pulp (10 to 30%, w/w) previously heat-treated and other additives such as flavorings (*see* **Note** 7).
13. Aseptically fill in appropriate packaging and, preferably, store under refrigeration.

6 Notes

1. Check the manufacturer's instructions for use in less than the recommended volume in the microbial culture envelope. More instructions are given in Chap. 5, in the **Notes** to Subheading 3.
2. Exopolysaccharide-producing probiotic culture is recommended to obtain better texture properties.
3. The chickpea extract must have a typical taste and odor, an absence of spoiling microorganisms, pathogens, and an absence of fermentation-inhibiting substances. The heat treatment must be carried out in such a way as to guarantee the safety of the product. If the heating is done in an open tank, at a lower temperature and longer time, an increase in the solids content of the aqueous extract will occur.
4. Generally, aqueous extracts are fermented to a pH of about 4.3. The fermentation time depends on the characteristics of the culture and temperature employed and typically ranges from 4 to 30 h.
5. Additional hygienic care must be taken in the draining step to avoid microbiological risk. It is recommended that this step be carried out under refrigeration. The whey released to obtain quark cheese has a high concentration of water-soluble components, such as carbohydrates, salts, and proteins, so it can be used to manufacture other food products.
6. Other thickening agents can be used to ensure the texture properties of the product, such as carrageenan gum (2–5%, w/w) and pectin (0.5–2%, w/w).
7. The acidity of fruit pulp can affect the viability of probiotic microorganisms during product storage. Therefore, perform feasibility tests to verify that the probiotic count is as desired.

References

1. Harper AR, Dobson RC, Morris VK, Moggré GJ (2022) Fermentation of plant-based dairy alternatives by lactic acid bacteria. Microbiol Biotechnol 15(5):1404–1421
2. Short EC, Kinchla AJ, Nolden AA (2021) Plant-based cheeses: a systematic review of sensory evaluation studies and strategies to increase consumer acceptance. Foods 10(4):72
3. Craig WJ, Mangels AR, Brothers CJ (2022) Nutritional profiles of non-dairy plant-based cheese alternatives. Nutrients 14(6):1247
4. Jeewanthi RKC, Paik HD (2018) Modifications of nutritional, structural, and sensory characteristics of non-dairy soy cheese analogs to improve their quality attributes. J Food Sci Technol 55(11):4384–4394
5. Mattice KD, Marangoni AG (2020) Physical properties of plant-based cheese products produced with zein. Food Hydrocoll 105:105746
6. Grossmann L, McClements DJ (2021) The science of plant-based foods: approaches to create nutritious and sustainable plant-based cheese analogs. Trends Food Sci Technol 118: 207–229
7. Fu W, Yano H (2020) Development of "new" bread and cheese. PRO 8(12):154

8. Bachmann HP (2001) Cheese analogues: a review. Int Dairy J 11(4–7):505–515
9. Guinee TP, Mulholland EO, Kelly J, Callaghan DJO (2007) Effect of protein-to-fat ratio of milk on the composition, manufacturing efficiency, and yield of cheddar cheese. J Dairy Sci 90:110–123
10. Kamath R, Basak S, Gokhale J (2022) Recent trends in the development of healthy and functional cheese analogues-a review. LWT 155: 112991
11. Masiá C, Jensen PE, Petersen IL, Buldo P (2022) Design of a Functional pea Protein Matrix for fermented plant-based cheese. Foods 11(2):178
12. Lam ACY, Can Karaca A, Tyler RT, Nickerson MT (2018) Pea protein isolates: structure, extraction, and functionality. Food Rev Int 34(2):126–147
13. Matias NS, Bedani R, Castro IA, Saad SMI (2014) A probiotic soy-based innovative product as an alternative to petit-suisse cheese. LWT 59(1):411–417
14. Liu K (2008) Food use of whole soybeans. In: Johnson LA, White PJ, Galloway R (eds) Soybeans. AOCS Press, Urbana
15. Faisal M, Mulana F, Alam PN, Daimon H (2014) Wastewater characteristics from tofu processing facilities in Banda Aceh. Proc Ann Int Conf Syiah Kuala University-Life Sciences & Engineering Chapter 4(1):18–21
16. Zeybekoglu N, Ozhan AH, Boyacioglu O (2021) Probiotic tofu with Bacillus clausii spores to support gastrointestinal microflora. Adnan Menderes Üniversitesi Sağlık Bilimleri Fakültesi Dergisi 5(3):534–545
17. Wolfschoon-Pombo AF, Dang BP, Chiriboga BC (2018) Forced syneresis determination results from commercial cream cheese samples. Int Dairy J 85:129–136
18. Liong MT, Easa AM, Lim PT et al (2009) Survival, growth characteristics and bioactive potential of Lactobacillus acidophilus in a soy-based cream cheese. J Sci Food Agric 89(8):1382–1391
19. Lim TJ, Easa AM, Karim AA et al (2011) Development of soy-based cream cheese via the addition of microbial transglutaminase, soy protein isolate and maltodextrin. Br Food J 113(9):1147–1172
20. Rinaldoni AN, Palatnik DR, Zaritzky N, Campderros ME (2014) Soft cheese-like product development enriched with soy protein concentrates. LWT Food Sci Technol 55(1): 139–147
21. Wolfschoon Pombo AF (2021) Cream cheese: historical, manufacturing, and physicochemical aspects. Int Dairy J 117:104948

Chapter 7

Probiotic Fruit Juices

Oscar O. Romero-Chapol, Abigail Varela-Pérez, Ana G. Castillo-Olmos, Hugo S. García, and Cynthia Cano-Sarmiento

Abstract

Regular consumption of probiotic bacteria is done mainly by ingestion of dairy or fermented foods; however, some health issues, changing lifestyles, and feeding habits deprive part of the population of their benefits. Therefore, consumption alternatives, such as fruit juices, represent a challenge attributed to the presence of intrinsic conditions that affect the viability of bacteria. In this chapter, we describe a method incorporating probiotic bacteria encapsulated in an alginate matrix using an emulsification process as a pretreatment into fruit juices. We also provide the reader with the techniques for morphological analysis by scanning electron microscopy, as well as the characterization of the juice and the evaluation of cell viability against simulated gastric conditions.

Key words Probiotics, Juice, Encapsulation, Viability, Scanning electron microscopy

1 Introduction

Currently, the demand for functional products formulated with probiotics has gained more importance due to their beneficial effects on several diseases and the advantages they provide by modulating immune responses and gut microbiota through several mechanisms [1–3]. Probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" [4]. Different foods have been fermented or inoculated with probiotics to be examined as potential carriers of these microorganisms; however, it must be considered that the beneficial effect on the host might be modified according to the probiotic strain or mixture of them that is used [5–7]. Some *Lactobacillus* and *Bifidobacterium* species are probiotics and the most commonly employed strains in several food products [6]. Traditionally, probiotics are added to fermented milk or other dairy products. However, it is also evaluated by adding it to other matrices such as juices, as some consumers are vegans, lactose intolerant, or have protein

Adriano Gomes da Cruz et al. (eds.), *Probiotic Foods and Beverages: Technologies and Protocols*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-3187-4_7,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2023

allergies. For this reason, probiotic fruit juices represent an adequate alternative to the preparation of non-dairy probiotic foods, in addition to supplying nutrients such as minerals, vitamins, fiber, and antioxidants. One of their main advantages is the pleasant taste for all age groups, resulting in a functional food that can be accepted by the public [8]. Fruit juices (100% fruit content), nectars (25–99% fruit), and juice drinks (up to 25% fruit content) have become crucial sectors in the food industry. Nevertheless, in the case of probiotic liquid matrices, fruit juices are preferentially employed since, unlike nectar and juice drinks, they do not contain food additives such as preservatives or sweeteners that allow this type of product to achieve a low price in the market but also carry a negative effect based on the excessive consumption of these sweeteners that can have on the health of consumers, leading to dysbiosis in gut microbiota and increased obesity risk [9, 10]. Fruit juices contain nutrients naturally beneficial to health, like calcium, retinol, vitamins, nicotinic acid, riboflavin, pantothenic acid, β-carotene, biotin, dietary fiber, anthocyanins, and antioxidants. Some of these compounds have demonstrated harmful effects against certain pathogenic microorganisms and, in contrast, promote the growth of bacteria with beneficial properties. Also, another attribute of juices is that they are readily digested in the stomach compared with a dairy matrix; thus, probiotic bacteria remain in the stomach's acid environment for a shorter time [1, 6, 11, 12]. Studies employing fruit juice to prepare probiotic foods have used the juices of pineapple, cranberry, strawberry, sweet lemon, mango, apple, peach, pomegranate, grape, orange, blackcurrant, banana, papaya, and pear, among others [11–13]. Specific bacteria from the genus *Lactobacillus*, such as *L. plantarum*, *L. acidophilus*, and *L. casei*, have proven greater acid tolerance for survival in strawberry, orange, and cranberry juices [14, 15].

There are bacteria that, when added to juices, can carry out a fermentation process, resulting in a reduction in sugar in the food and a high rate of bacterial survival because of their adaptation to the media. In addition, metabolites are produced during fermentation that help increase the product's attributes, including bacteriocins which impede contamination during storage. However, even though fruits are an adequate matrix for probiotic growth, the viability of the probiotics in the juice is more complicated in comparison to a dairy matrix because of the requirement for lactic acid bacteria to shelter from the acid environment of fruits, along with not containing enough peptides and free amino acids present for the metabolism of a probiotic culture [1, 13]. Elements that influence probiotic viability include intrinsic matrix factors such as titratable acidity, pH value, molecular oxygen, water activity, artificial flavors and colorings, chemical or microbial agents, and preservatives like hydrogen peroxide and bacteriocins. On the other hand, the type of probiotic strain, compatibility of different strains,

percentage of inoculum, and the stress bacteria suffer throughout the gastrointestinal tract. In general, fruits have a low pH and high quantities of organic acids, which increase in concentration in non-dissociated form; hence the survival of probiotic bacteria is defined by the highly acidic environment and the intrinsic antimicrobial activity of the organic acids that accumulate. For example, *Lactobacilli* resist and survive better than *Bifidobacteria* in fruit juices, with a pH value of 3.7 to 4.3. Moreover, other parameters also affect probiotic survival during production, processing, and manipulation: a thermal sterilization process, pasteurization, cooling rate, volume, packaging material, and storage temperature [11–13].

An essential factor in the manufacturing of fruit juices is the method of sterilization, which can be divided into thermal and non-thermal; non-thermal methods of sterilization employ pulsed electric fields, high-pressure processes, ultrasound, and ionizing radiation, all of which achieve the inactivation of microorganisms at temperatures between 40 and 55 °C [16]. On the other hand, thermal methods of pasteurization are applied at temperatures between 60 and 90 °C from a few seconds to some minutes [17].

Pasteurization is a heat process that increases a food's shelf life, inactivating certain enzymes and microorganisms (yeasts, molds, and bacteria) while producing a loss of flavor and changes in composition. As a result, primary properties like food taste are frequently modified. However, this technique has been refined, so fewer properties are modified in the final product; da Costa et al. [18] prepared orange juice pasteurized at 80 °C for 20 min, cooled to 37 °C, and added oligofructose before fermentation with *Lactobacillus paracasei* ssp. *paracasei*, the juice was stored at 4 °C for 28 days, and its physicochemical and sensory properties were evaluated. The authors reported that it was possible to develop symbiotic cultures in orange juice added with oligofructose (a prebiotic) and probiotics without changing the physicochemical characteristics and acceptance of pure juice, despite using pasteurization.

On the other hand, the main objective of high-pressure sterilization is to maintain the viability of probiotic bacteria while eliminating possible pathogens present in the food matrix. However, this process induces alterations in the cell membrane and morphology, inhibits the synthesis of proteins and enzymes, and affects the interruption of translation and transcription, in addition to the functions of reproduction and survival in the bacteria of interest [16]. The effectiveness of high-pressure homogenization treatments depends on the pressure, time, number of cycles, and temperature. However, food contains carbohydrates, vitamins, and minerals that can be susceptible to high pressure. Hence, the design and the development of probiotic juice turns into a comprehensive

task that needs to consider technical and microbial aspects without affecting or having the most negligible possible effect on the quality, nutrient composition, and functionality of the final food product. Accordingly, Oliveira et al. [19] developed a juice mix from mango and carrot, which was processed both thermally and with high hydrostatic pressure and supplemented with *L. plantarum*. Bacteria caused a reduction of pH and increased acidity in juices but did not modify their antioxidant capacity or the α- and β-carotene content. Specifically, the high-energy process maintained microbiological quality, unlike pasteurized juices with high psychrotrophic counts after 35 days of storage. Processing juice at high pressures proved to be more effective than the thermal process, with fewer modifications in composition, while maintaining the excellent viability of *L. plantarum* [19].

Finally, microorganisms can be inactivated by sterilizing with electric pulses by applying high-voltage pulses to liquid or semi-liquid foods placed between two electrodes with treatments from micro to milliseconds. This leads to the formation of pores in the microbial cell membrane that entails a rupture of the cell, which causes the release of cell contents and intrusion of surrounding media [16].

In light of a critical risk of juice viability being lost due to previously described variables, bacteria can be protected by encapsulation and subsequently added to the food matrix. With this, survival rates can be conserved during all the processes involved in encapsulation and throughout its shelf life [20]. However, supplementation of probiotic bacteria inside a liquid food matrix, as in the case of fruit juices, represents a technological challenge where both factors need to be favored by the mixture. On one side, it is necessary to protect probiotic bacteria against the intrinsic factors of juice that affect cell survival and, consequently, their beneficial effects in the host, while on the side of the food matrix, the growth of the bacterial population might cause damage due to CO_2 production and alterations in sensory factors like taste, texture, and color [21]. Depending on the technique used for encapsulation, viability varies, with reported percentages ranging between 80 and 95% [22]. Additionally, fewer modifications to juice composition can be achieved through encapsulation.

Table 1 shows a compilation of some techniques, materials, and bacteria used to develop probiotic juices and the results of applying these technologies.

The ionic gelation process that uses alginate as an encapsulant matrix is one of the techniques mainly employed for the development of probiotic juices because of its compatibility with the components present in the mixture, high viability rate during storage, and, finally, for preserving the sensory properties of juice [2, 25].

Table 1
Juice with encapsulated and non-encapsulated probiotics

Juice	Bacteria used	Encapsulation technique and wall material	Results	References
Pineapple, orange, raspberry	*L. casei* DSM 20011	Vibration technology; sodium alginate	Preservation of viability of encapsulated bacteria at 28 days of storage in raspberry and orange juice compared with free cells; ~ 57% viability for encapsulated systems and < 15% in free systems	[15]
Apple, orange	*L. rhamnosus*, *B. longum*, *L. salivarius*, *L. plantarum*, *L. acidophilus*, *L. paracasei*, *B. lactis* type Bi-04, *B. lactis* Bi-07	Emulsification and ionic gelation; sodium alginate	Viability in the juice after 6 weeks of storage of ~60 and 64% for encapsulated systems; no viability for free cells. Encapsulated groups present less diminution of pH	[23]
Apple	*L. rhamnosus* GG	Emulsification and ionic gelation; sodium alginate	Viability of encapsulated probiotic during storage of ~100 and ~ 80% in case of free cell administration	[2]
Grape	*L. acidophilus* PTCC 1643, *B. bifidum* PTCC 1644	Emulsification and ionic gelation; sodium alginate	Sensory aspects like acidity, color, turbidity, and soluble solids are preserved better in encapsulated systems. Viability after storage of ~88% in encapsulated systems and ~ 78% in free cells	[24]
Apple	*L. rhamnosus* GG	Extrusion and ionic gelation; sodium alginate and alginate–inulin	Viability of encapsulated bacteria during storage of ~80 and ~ 20% in case of free cells. More excellent score in sensory evaluation (~ 1 point) of juices with encapsulated bacteria	[25]

2 Case Study

2.1 Materials/ Reagents

The use of distilled water sterilized by autoclave at a pressure of 10 psi and temperature of 115 °C is recommended, as well as the use of reagent-grade components for the preparation of all solutio/ ns. Reagents must be prepared at room temperature (~ 25 °C); temperature control is not necessary for most solutions. All material inoculated with bacteria must be sterilized before its disposal. In the case of culture media (MRS broth, MRS agar, and Gomori buffer), previous sterilization is mandatory to avoid contamination.

2.2 Cell Culture

Freeze-dried or frozen cells of probiotic strains are reactivated at least three times before use. Probiotic bacteria of the genus *Lactobacillus* must be inoculated at 1% v/v in MRS broth at 37 °C and pH 5.9 for 24 h at 150 rpm [3]. In the case of probiotic bacteria of the genus *Bifidobacterium*, supplement media with 0.05% w/v L-cysteine is recommended (*see* **Note 1**) [26].

2.3 Encapsulant Mixture

A bacterial suspension is obtained by centrifugation at 10,000 × *g* and 4 °C for 10 min. Two washes with 0.9% w/v saline solution (NaCl) must be conducted before resuspension at the same volume. A mix of 25 mL of 10 log CFU/mL bacterial suspension with 100 mL of 3% w/v sodium alginate, 100 mL of canola vegetable oil, and 1 mL of Tween® 80 has to be prepared. The mixture must be homogenized at 4000 rpm for 7.5 min with an Ultra-turrax® model T-25 rotor-stator type homogenizer (IKA, Staufen, Germany).

2.4 Cell Viability (Buffers)

A Gomori buffer (phosphate buffer) is prepared for the liberation and quantification of encapsulated bacteria. For example, to prepare 0.1 M K_2HPO_4 and KH_2PO_4 stock solutions at pH 7 and 25 °C, it is necessary to mix 61.5 mL of K_2HPO_4 and 38.5 mL of KH_2PO_4. The buffer is left to rest for 24 h at 4 °C before use.

2.5 Gastrointestinal Solutions

Enzyme concentrations are 75 U/mL α-amylase, 2000 U/mL pepsin, 60 U/mL gastric lipase, 100 U/mL pancreatin, and 10 mM bile solution. Solutions employed to modify pH throughout the simulation are HCl and NaOH at 1 M; in each stage, different volumes of 0.3 M $CaCl_2$ are added. At every stage of the simulation, one phase is used; saliva solution must have concentrations of 15.1 mM KCl, 3.7 mM KH_2PO_4, 13.6 mM $NaHCO_3$, 0.15 mM $MgCl_2(H_2O)_6$, 0.06 mM $(NH_4)_2CO_3$, 1.1 mM HCl, and 1.5 mM $CaCl_2(H_2O)_2$; gastric solution needs to have concentrations of 6.9 mM KCl, 0.9 mM KH_2PO_4, 25 mM $NaHCO_3$, 47.2 mM NaCl, 0.12 mM $MgCl_2(H_2O)_6$, 0.5 mM $(NH_4)_2CO_3$, 15.6 mM HCl, and 0.15 mM $CaCl_2(H_2O)_2$; finally, the small intestine solution must have concentrations of 6.8 mM KCl, 0.8 mM KH_2PO_4, 85 mM $NaHCO_3$, 38.4 mM NaCl, 0.33 mM $MgCl_2(H_2O)_6$, 8.4 mM HCl, and 0.6 mM $CaCl_2(H_2O)_2$.

3 Methods

3.1 Encapsulation Process

Encapsulant mixture is dispersed in 0.1 M $CaCl_2$ solution by dripping it with a 21 G needle placed 15 cm above the solution at 2.5 mL/min, employing a peristaltic pump coupled with a head and a silicone hose; once capsules are formed, they are stored in 0.1 M $CaCl_2$ solution for 12 h at 4 °C to promote greater reticulation (*see* **Note 2**).

3.1.1 Viability Assay

Viability and encapsulation efficiency is evaluated by dissolving 1 g of capsules in Gomori buffer for 5 min with stirring at 2000 rpm with a vortex; from this solution, 100 μL are used as inoculum, serial decimal dilutions are done, and then the mixture is cultured in MRS agar for subsequent counting (*see* **Note 3**). In the case of free cells, buffer and agitation are omitted.

3.2 Inoculation and Characterization of Fruit Juices with Encapsulated Probiotic

Juice is inoculated with 10% w/v probiotic capsules (*see* **Notes 4** and **5**) [27].

3.2.1 Characterization of Probiotic Juice

Each week the CIELab scale parameters L*, a*, and b* are determined (*see* **Note 6**) with a colorimeter to determine the total change of color (*see* **Note 7**). The pH and amount of soluble solids are monitored weekly (*see* **Note 6**) using a pH meter and a refractometer.

Interpretation of the color space might help to illustrate in visual form the change of color over time (*see* Fig. 1).

4 In Vitro Evaluation

Evaluation of in vitro gastrointestinal simulation is carried out employing the COST INFOGEST methodology proposed for in vitro digestion [28, 29], where three stages of digestion are simulated: oral, gastric, and intestinal.

Capsules (5 g) and/or free cells (5 mL) are mixed with 4 mL of salivary solution, 0.75 mL of α-amylase solution, and 25 μL of $CaCl_2$; the pH is adjusted to 7 if required, and the mixture made up to 10 mL with ultrapure type I water. The mixture is incubated for 2 min at 37 °C. Once the incubation period is over, the reaction is finished by placing the mixture in an ice bath to slow down enzyme activity.

To the resultant salivary phase mixture, 8 mL of gastric solution, 0.667 mL of pepsin solution, 0.48 mL of gastric lipase, and 5 μL of $CaCl_2$ are added to form the gastric phase; after adding this, the pH is adjusted to 3.0 ± 0.2 and the solution made up to 20 mL

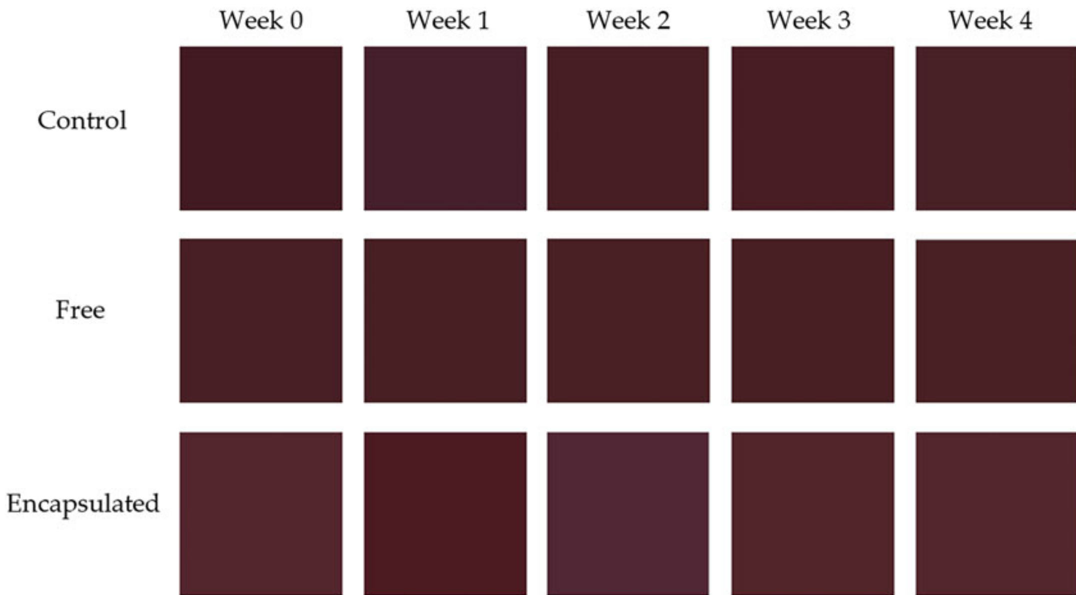

Fig. 1 CIELab space color changes in probiotic pomegranate juice
Evaluation of color of pomegranate juice during storage. "Control" sample is simple pomegranate juice, "Free" contains pomegranate juice with *Lacticaseibacillus rhamnosus* GG (LGG), and "Encapsulated" is pomegranate juice with capsules charged with LGG, which were elaborated by high-energy processes

with ultrapure type I water. Once the gastric phase is ready, it is incubated at 37 °C for 2 h. Following incubation, samples are cooled in the same way as the salivary phase in an ice bath.

In brief, to obtain the intestinal phase, 8 mL of small intestine solution, 5 mL of pancreatin, 40 μL of $CaCl_2$, and 3 mL of bile solution are mixed along with solution derived from the gastric phase; for intestinal phase simulation, the pH is adjusted to 7.0 ± 0.2 and the solution made up to 40 mL with ultrapure type I water. Then, the intestinal phase is incubated for 2 h at 37 °C with agitation at 95 rpm.

At the end of each stage of gastrointestinal simulation, aliquots are taken to conduct morphological analysis by microscopy (*see* Fig. 2) and viability through culture in Petri dishes.

5 Morphological Analysis

Scanning electron microscopy (SEM) is used to visualize morphological changes that the capsules may present initially or when subjected to various external factors, allowing these changes to be appreciated in greater detail. The same microscopy technique can be used to observe bacteria. In addition to SEM, the morphology of the capsules can be monitored through different morphometric parameters.

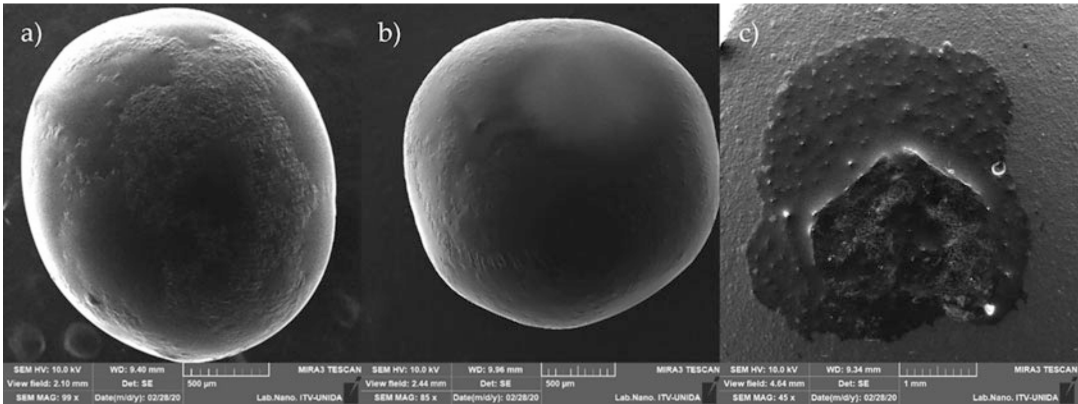

Fig. 2 SEM visualization of microcapsules during in vitro digestion
Capsules elaborated with high-energy techniques; (**a**) oral phase, (**b**) gastric phase, and (**c**) intestinal phase

5.1 Sampling, Preparation, and SEM Visualization

Morphological analysis is applied to capsules once they are prepared, during shelf life, and at the end of each gastrointestinal digestion stage. In all cases, one or two capsules are dried for 1 h in a laminar flow cabinet at 25 °C; the same drying method is applied for free bacteria, with the difference that the sample is taken directly from a colony in a Petri dish. Capsules and cells are dried once they are deposited on a stub with carbon tape. The conditions usually employed for SEM analysis are 10 and 8 kV voltages, a low vacuum, a secondary electron detector, and a work distance of 10 mm (*see* Fig. 2) (*see* **Note 8**).

5.2 Preparation and Visualization of Capsules with Morphometric Parameters

Morphometric parameters like Feret diameter, area, and circularity are obtained through photographs in JPG format taken with a professional camera at 15 cm from the objective with a resolution of 24.5 megapixels; for that, a medium containing 100 capsules is taken and placed in a Petri dish with millimetric paper (*see* Fig. 3), and they are analyzed through the ImageJ software (*see* **Note 9**).

6 Notes

1. The optimum growth temperature for most probiotic strains (predominantly *Lactobacillus*) is between 30 and 40 °C; however, 37 °C is recommended along with media with a pH range between 5.5 and 6.2. To promote an adequate mass exchange, the use of an incubator with agitation is recommended. In the case of *Bifidobacterium*, the range of pH might vary between 6.5 and 7.0 because of its lower acid tolerance [30].
2. It is possible to carry out an alternative method of dosage to obtain a smaller particle size by employing atomization through a SUJ1A configuration nozzle with a flux of CO_2 at 10 SCFH (standard cubic feet per hour) and 50 psi of pressure,

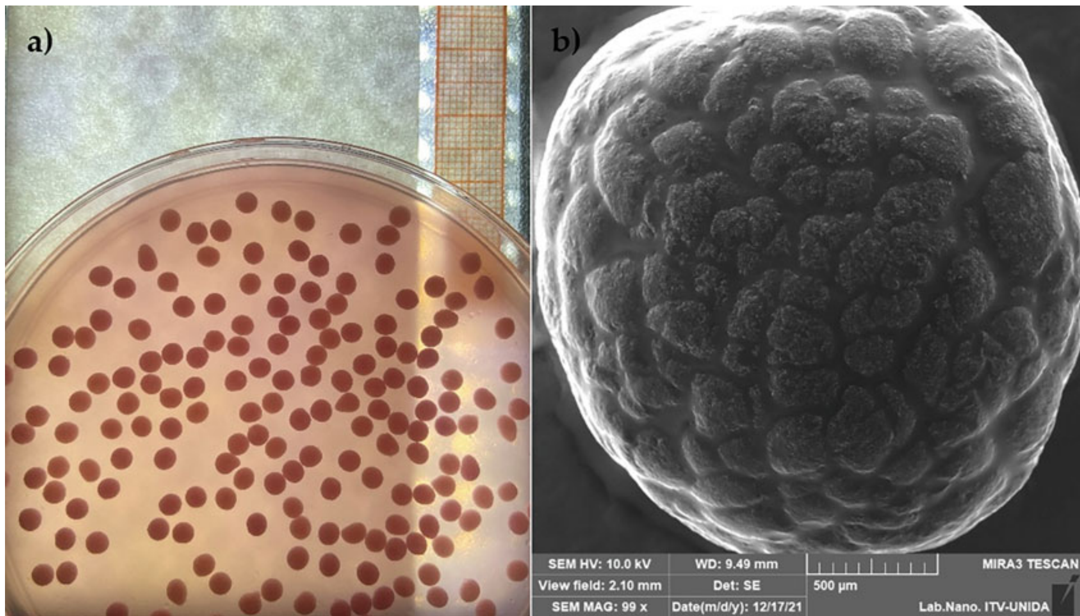

Fig. 3 Preparation of microcapsules for SEM visualization and digital image analysis
Preparation of capsules for determination of morphometric parameters via (**a**) digital camera and (**b**) scanning electron microscopy. Capsules obtained by high-energy techniques

15 cm work distance, and a $CaCl_2$ solution in gentle stirring. This method needs to filter capsules with a vacuum pump and 11 μm porous size filter paper before viability analysis.

3. Viability is evaluated with the following equation:

$$Viability\,(\%) = \frac{N}{N_0} \times 100$$

where viability is given as a percentage, N_0 is the number of bacteria (log CFU/mL) before encapsulation, and N is the number of bacteria (log CFU/mL) released by the capsules [2].

4. Clarified juices are recommended for product development; to facilitate analysis of cell viability; the volume of juice used depends on the beneficial effect sought to generate a functional food. Chelating agents in juices can destabilize the capsule wall, causing loss of viability by the interchange of Ca^+ with Na^+ and K^+ ions in juice [31].
5. Selecting a food matrix (juice) with beneficial properties is recommended to generate a functional food. The volume of juice must be based on existing studies that show their effectiveness. To obtain the beneficial effect of specific probiotic bacteria, it is necessary to have a cell concentration in food of between 6 and 8 log CFU/g [32].

6. It is recommended that all characterization analysis of the food matrix is done once viability has been assessed to avoid contamination. In addition, the frequency with which the food matrix is analyzed should provide kinetic data on viability and food evolution.

7. The change of color is analyzed with the following equation:

$$\Delta E_{ab} = \sqrt{(L_2 - L_1)^2 + (a_2 - a_1)^2 + (b_2 - b_1)^2}$$

   Where L_1 and L_2 are the luminosity of samples 1 and 2, a_1 and a_2 are the red/green coordinates of samples 1 and 2, and b_1 and b_2 are the yellow/blue coordinates of samples 1 and 2. Sample 1 refers to the control parameters of juice at time 0 of storage [33]. To obtain adequate measures of color, it is recommended to use a vessel that fits with the colorimeter sensor and has dark walls and a lid to avoid the passage of external light through the colorimeter sensor.

8. The conditions used for visualization of bacteria through SEM as well as the drying period of the sample vary in agreement with each bacterial strain; if bacteria tend to have large amounts of mucin, extending the drying time up to 8 h is recommended, besides using another method of drying such as desiccator drying.

9. The circularity of capsules is calculated from the following equation:

$$Circularity = \frac{4\pi A}{P^{2.}}$$

   Where A is the capsule area and P is the perimeter; if values of circularity area are equal or close to 1, capsules are considered to have a morphology geometrically comparable to a perfect circle [34]. The image analysis software (ImageJ) directly provides parameters like Feret diameter and area.

References

1. Fernandes P, Rodriguez S (2018) Turning fruit juice into probiotic beverages. In: Rajauria G, Tiwari BK (eds) Fruit juices, 1st edn. Academic Press, Cambridge
2. Romero-Chapol OO, Varela-Pérez A, Castillo-Olmos AG et al (2022) Encapsulation of *Lacticaseibacillus rhamnosus* GG: probiotic survival, in vitro digestion and viability in apple juice and yogurt. Appl Sci 12(4):2141
3. Varela-Pérez A, Romero-Chapol OO, Castillo-Olmos AG et al (2022) Encapsulation of *lactobacillus gasseri*: characterization, probiotic survival, in vitro evaluation and viability in apple juice. Foods 11(5):740
4. Hill C, Guarner F, Reid G et al (2014) The international scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol 11:506–514. https://doi.org/10.1038/nrgastro.2014.66
5. Lee E, Song E, Nam Y et al (2018) Probiotics in human health and disease: from nutribiotics to pharmabiotics. J Microbiol 56(11):773–782

6. Nguyen BT, Bujna E, Fekete N et al (2019) Probiotic beverage from pineapple juice fermented with *lactobacillus* and *Bifidobacterium* strains. Front Nutr 6(54):1–7
7. Rodrigues FJ, Cedran MF, Bicas JL et al (2020) Encapsulated probiotic cells: relevant techniques, natural sources as encapsulating materials and food applications – a narrative review. Food Res Int 137:1–16. https://doi.org/10.1016/j.foodres.2020.109682
8. Nematollahi A, Sohrabvandi S, Mortazavian AM et al (2016) Viability of probiotic bacteria and some chemical and sensory characteristics in cornelian cherry juice during cold storage. Electron J Biotechnol 21:49–53. https://doi.org/10.1016/j.ejbt.2016.03.001
9. Neves MF, Trombin VG, Lopes FF et al (2011) Definition of juice, nectar and still drink. In: The orange juice business. Wageningen Academic Publishers, Wageningen
10. Sánchez-Tapia M, Miller AW, Granados-Portillo O et al (2020) The development of metabolic endotoxemia is dependent on the type of sweetener and the presence of saturated fat in the diet. Gut Microbes 12:1–19. https://doi.org/10.1080/19490976.2020.1801301
11. Patel AR (2017) Probiotic fruit and vegetable juices- recent advances and future perspective. Int Food Res J 24:1850–1857
12. Žuntar I, Petric Z, Bursać Kovačević D et al (2020) Safety of probiotics: functional fruit beverages and nutraceuticals. Foods 9(7):947
13. Aspri M, Papademas P, Tsaltas D (2020) Review on non-dairy probiotics and their use in non-dairy based products. Fermentation 6(1):30
14. Perricone M, Bevilacqua A, Altieri C et al (2015) Challenges for the production of probiotic fruit juices. Beverages 1(2):95–103
15. Olivares A, Soto C, Caballero E et al (2019) Survival of microencapsulated *lactobacillus casei* (prepared by vibration technology) in fruit juice during cold storage. Electron J Biotechnol 42:42–48. https://doi.org/10.1016/j.ejbt.2019.10.002
16. Asaithambi N, Singh SK, Singha P (2021) Current status of non-thermal processing of probiotic foods: a review. J Food Eng 303:110567. https://doi.org/10.1016/j.jfoodeng.2021.110567
17. Benjamin O, Gamrasni D (2020) Microbial, nutritional, and organoleptic quality of pomegranate juice following high-pressure homogenization and low-temperature pasteurization. J Food Sci 85(3):592–599
18. da Costa GM, de Carvalho Silva JV, Mingotti JD et al (2017) Effect of ascorbic acid or oligofructose supplementation on *L. paracasei* viability, physicochemical characteristics and acceptance of probiotic orange juice. LWT Food Sci Technol 75:195–220. https://doi.org/10.1016/j.lwt.2016.08.051
19. Oliveira PMD, Leite Júnior BRDC, Martins EMF et al (2020) Impact of high pressure and thermal processing on probiotic mixed mango and carrot juices. J Food Process Preserv 44(7): 1–11
20. Betoret N, Puente L, Díaz MJ et al (2003) Development of probiotic-enriched dried fruits by vacuum impregnation. J Food Eng 56:273–277. https://doi.org/10.1016/S0260-8774(02)00268-6
21. Ephrem E, Najjar A, Charcosset C et al (2018) Encapsulation of natural active compounds, enzymes, and probiotics for fruit juice fortification, preservation, and processing: an overview. J Funct Foods 48:65–84. https://doi.org/10.1016/j.jff.2018.06.021
22. Krasaekoopt W, Bhandari B, Deeth H (2003) Evaluation of encapsulation techniques of probiotics for yoghurt. Int Dairy J 13:3–13. https://doi.org/10.1016/S0958-6946(02)00155-3
23. Ding WK, Shah NP (2008) Survival of free and microencapsulated probiotic bacteria in orange and apple juices. Int Food Res J 15(2): 219–232
24. Mokhtari S, Jafari SM, Khomeiri M (2018) Survival of encapsulated probiotics in pasteurized grape juice and evaluation of their properties during storage. Food Sci Technol Int 25(2):120–129
25. Gandomi H, Abbaszadeh S, Misaghi A et al (2016) Effect of chitosan-alginate encapsulation with inulin on survival of *lactobacillus rhamnosus* GG during apple juice storage and under simulated gastrointestinal conditions. LWT Food Sci Technol 69:365–371. https://doi.org/10.1016/j.lwt.2016.01.064
26. Alehosseini A, Gomez del Pulgar E, Fabra MJ et al (2019) Agarose-based freeze-dried capsules prepared by the oil-induced biphasic hydrogel particle formation approach for the protection of sensitive probiotic bacteria. Food Hydrocoll 87:487–496. https://doi.org/10.1016/j.foodhyd.2018.08.032
27. Chaikham P (2015) Stability of probiotics encapsulated with Thai herbal extracts in fruit juices and yoghurt during refrigerated storage. Food Biosci 12(1):61–66

28. Minekus M, Alminger M, Alvito P et al (2014) A standardized static *in vitro* digestion method suitable for food - an international consensus. Food Funct 5(6):1113–1124
29. Brodkorb A, Egger L, Alminger M et al (2019) INFOGEST static in vitro simulation of gastrointestinal food digestion. Nat Protoc 14:991–1014. https://doi.org/10.1038/s41596-018-0119-1
30. Grattepanche F, Lacroix C (2013) Production of viable probiotic cells. In: McNeil B, Archer D, Giavasis I, Harvey L (eds) Microbial production of food ingredients, enzymes and nutraceuticals, 1st edn. Woodhead Publishing, Sawston
31. Nualkaekul S, Lenton D, Cook MT et al (2012) Chitosan coated alginate beads for the survival of microencapsulated *lactobacillus plantarum* in pomegranate juice. Carbohydr Polym 90(3):1281–1287
32. Senadeera SS, Prasanna PHP, Jayawardana NWIA et al (2018) Antioxidant, physicochemical, microbiological, and sensory properties of probiotic yoghurt incorporated with various *Annona* species pulp. Heliyon 4(11):1–18
33. Juárez-Trujillo N, Jiménez-Fernández M, Franco-Robles E et al (2020) Effect of three-stage encapsulation on survival of emulsified *Bifidobacterium animalis* subsp. *lactis* during processing, storage and simulated gastrointestinal tests. LWT food. Sci Technol 137:110468
34. Dorożyński PP, Kulinowski P, Mendyk A et al (2010) Novel application of MRI technique combined with flow-through cell dissolution apparatus as supportive discriminatory test for evaluation of controlled release formulations. AAPS PharmSciTech 11(2):588–597

Chapter 8

Probiotic Fermented Vegetables

Hadi Pourjafar, Tatiana Colombo Pimentel, and Tahis R. Baú

Abstract

Fermented foods (as functional foods) are rich in various nutrients that benefit consumers' health. Fermented vegetables are a group of fermented foods that have a long history and have been used by humans in the distant past until today as healthy, delicious, and popular foods, and most importantly, with a long shelf life. Probiotic fermented vegetables are products that have been fermented and produced by probiotics; either these microorganisms are naturally and intrinsically present in the vegetables or added manually. In some cases, despite probiotics in fermented vegetables, the primary fermentation process is carried out by non-probiotic microorganisms, and probiotics' role in this process is minor. But in any case, it acts as a carrier for probiotics to be transferred to the consumer's body and digestive system. Therefore, fermented vegetables are an excellent source for isolating and commercializing different and beneficial probiotic strains. Here we will describe the process of obtaining probiotic vegetable pickles, probiotic sauerkraut, and probiotic natto.

Key words Fermented vegetables, Health-promoting, Probiotic vegetable pickles, Probiotic sauerkraut, Probiotic natto

1 Introduction

Functional food products, especially fermented foods, are products that have one or more positive effects on health yonder a rudimentary diet [1, 2]. Meanwhile, the health role of fermented probiotic products is also prominent, and extensive studies have been conducted in this field. As a result, the probiotic product market is growing very rapidly owing to enlarged customer consciousness about the impact of these products on health. Currently, probiotic products, especially probiotic fermented food products, comprise 60–70% of the total functional food market [3–5]. The worldwide shop for probiotic products was around 24.8 billion € in 2011, above 31.1 billion € in 2015, and is anticipated to reach around 43 billion € by 2020 [6]. Today, non-dairy fermented probiotic products have received more attention than dairy products due to

Adriano Gomes da Cruz et al. (eds.), *Probiotic Foods and Beverages: Technologies and Protocols*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-3187-4_8,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2023

their low fat and more straightforward production process. In addition, they are used for people with allergies to dairy products. Therefore, it has more demand from consumers [6–8].

The dietetic and biological potential of vegetables has led to their alteration into products with multiple characteristics in upholding the steadiness of microorganisms. Vegetables are rich in vitamins, minerals, and valuable fibers and usually cause health effects on the consumer's body. Many of them are even used to treat various diseases. Suppose these products are fermented by probiotic microorganisms, in addition to the presence of probiotics inside these products, which are transferred to the consumer's body either completely or as paraprobiotics and postbiotics, some vegetable compounds are fermented and converted into healthier components for the consumer's body. These can be referred to as beneficial organic acids, such as gluconic and glucuronic acids. Studies have revealed that alkalis, bioflavonoids, potassium salts, and vitamins in vegetables can positively impact the inhibition and cure of cardiovascular illness [8–11].

Furthermore, it has been revealed that the valuable properties of vegetables can be enriched via a natural procedure like lactic fermentation. Currently, scientists are investigating the lactic fermentation of vegetables as a natural preservation process. Furthermore, various vegetables have natural prebiotic ingredients that encourage the growth of certain probiotic microorganisms in the fermented food product and gastrointestinal tract [12].

Many studies have been done concerning probiotic fermented dairy products, and most of the products available and accessible to consumers are mostly dairy products. However, many consumers are looking for a better alternative to dairy products due to some disadvantages, such as people's high-fat content and lactose intolerance. Hence, the consumption of probiotic fermented vegetable foods can be a worthy substitute for some people, such as vegetarians and people with allergic reactions to milk proteins [6, 7].

The fermentation process is an old method to increase the shelf life of vegetable products, which happen to be highly perishable. Good examples of old and well-known fermented vegetables include products such as Chinese "PaoCai," Korean "Kimchi" (sauerkraut/sour cabbage), Indonesian "Tempeh," Japanese "Natto" and "Miso" (fermented soybeans), Chinese "Jiangshui" (Chinese cabbage, potherbs, radish sprouts, mustard, and fermented celery), which their health-promoting effects have been proven. Moreover, various probiotic strains have been isolated from them [13]. In general, two modes can be considered for probiotic fermented vegetables; one is fermented products that only act as a carrier for probiotics, and the fermentation process is mainly done by other microorganisms (non-probiotic microorganisms). The second case is products that are fermented by probiotic strains. However, separating these two modes in natural probiotic

fermented products is difficult unless one or two specific probiotic strains are intentionally added to produce commercial probiotic fermented foods. Probiotic vegetables can be manufactured by adding probiotics, e.g., into the vegetables/juice or fermentation with the probiotic microorganism. The second way (fermentation) is more valued because the probiotic grows into the vegetable texture/juice into a more adapted probiotic and a low-sugar manufactured good, possibly improving its viability [14, 15]. Furthermore, during fermentation, some metabolites of probiotic microorganisms, for instance, bacteriocins, exopolysaccharides, and bacterial cellulose, can increase the quality of the probiotic fermented product and upsurge their shelf life over the storage period [6].

A noteworthy point in fermented vegetables is the high acidity and a significant drop in pH in some of these products compared to fermented dairy products. As a result, probiotic strains should be used in these products if they are resistant to acidic conditions (pH ~ 3.5) [7]. It has been revealed that in fermented vegetables (pH 3.7–4.3), *Lactobacilli* can struggle and survive better than *Bifidobacteria* [16]. However, some vegetables may have ingredients that sustain the viability of probiotics, such as saccharides and organic acids that may be used as a carbon source, ascorbic acid that drops O/R potential, and cellulose that can protect the probiotics during the fermentation process and storage time [17]. Cabbage, soybean, beetroot, carrot, and celery are some instances of vegetables used as substrates for the transfer of probiotics. Various probiotic strains, regular species of *Lactobacillus* and *Bifidobacterium*, such as *Lactobacillus acidophilus*, *L. casei, L. paracasei, L. rhamnosus*, *L. fermentum*, *L. plantarum*, and *Bifidobacterium bifidum*, *B. breve*, *B. longum* have been primarily employed in the development of various probiotic fermented vegetables [6, 7].

It has been investigated that the suitability of cabbage juice [18, 19], tomato [20], and beet juice [21] using *L. acidophilus*, *L. casei*, *L. plantarum*, and *L. delbrueckii* after 4 weeks of storage at 4 °C and the amount of the active four probiotics in all fermented products ranged from 10^5–10^8 CFU/mL. In another study, Kun et al. (2008) disclosed that carrot juice can encourage the growth of *B. lactis* Bb-12, *B. bifidum* B7, and *B. bifidum* B 3.2. Entire probiotics displayed cell counts of 10^{10} CFU/mL [22]. In a study by Zhu et al. (2020), the survivability of *L. sanfranciscensis* in tomatoes was examined during 4 weeks of storage at 4 °C, and results indicated that the survival rate of *L. sanfranciscensis* in samples was $>10^6$–10^7 CFU/mL at the end of storing period [23].

One of the most critical fermented plant products with many fans and good health effects is fermented soybean, from which several strains of probiotics have been isolated, and some have even been commercialized. For example, it has been determined that in the tempeh, traditional Indonesian soybean cake, *Rhizopus*

microsporus var. oligosporus IFO 8631, a high concentration of Gamma-Aminobutyric Acid/GABA (GABA is the primary inhibitory neurotransmitter in the central nervous system/CNS. Its primary role is decreasing neuronal excitability all over the nervous system. It reduces a nerve cell's capability to receive, make, or send chemical messages to other nerve cells. Various homeopathic situations are related to the alteration levels of GABA. Manifold medicines that target the GABA receptor) were detected [24]. In addition, some fermented soybeans, such as Japanese Miso and Malaysian fermented soybean, are a source of probiotic yeast and lactic acid bacteria/LAB like *Zygosaccharomyces sapae* I-6 L and *L. plantarum* LAB12, respectively, which have anti-inflammatory properties [25, 26]. Various investigations have shown that many probiotic strains isolated from fermented soybean products belong to the *Bacillus* genus. For example, *Bacillus licheniformis* 141 as a probiotic, isolated from Cheonggukjang, a Korean traditional soybean paste, which increases the lifecycle of *Caenorhabditis elegans* nematode over serotonin signaling; *Bacillus paralichemiformis* and *Bacillus sonorensis* isolated from fermented soybean paste that ameliorated obesity, Nonalcoholic Fatty Liver Disease/ NAFLD and insulin resistance in a mice model of obesity [13, 27, 28]. Therefore, various plant products, especially fermented soybean products, can be an important source of bacterial and yeast probiotics. In Table 1 some probiotic fermented vegetable products are presented. This chapter describes the processes for obtaining probiotic vegetable pickles (*see* Subheading 2), probiotic sauerkraut (*see* Subheading 3), and probiotic natto (*see* Subheading 4).

2 Probiotic Vegetable Pickles

Probiotic vegetable pickles are products obtained by fermenting vegetables, producing acids in a brine initially composed of water, sugar, and salt. Although the use of microorganisms claiming probiotics in pickled vegetables is still scarce, this product may be a promising source of probiotic microorganisms. The main materials and processing steps for obtaining probiotic vegetable pickles are described below:

2.1 Preparation of Probiotic Vegetable Pickles

2.1.1 Materials

1. Vegetable raw materials (e.g., cucumber, carrot, pepper, and others).
2. Drinking water.
3. Chlorine-based sanitizer (e.g., sodium hypochlorite or sodium dichloroisocyanurate).
4. Salt.
5. Glucose or sucrose.

Table 1
Some selected probiotic fermented vegetable products

Fermented vegetable product	Isolated possible probiotic strains	Health-promoting effects	Reference/s
Sauerkraut (Croatian)	*Lactobacillus brevis* SF15 *L. paraplantarum* SF9	Stimulation and regulation of the immune system	[29, 30]
Sauerkraut (Chinese, PaoCai)	*L. plantarum* LAP6, *L. plantarum* S2-5, *L. plantarum* P2, *L. plantarum* S4-1, *Pediococcus pentosaceus* MP12	Prevention of Salmonellosis, serum cholesterol reduction, immunomodulation (stimulation of Th1 type cytokines)	[31–33]
Sauerkraut (Indonesian)	*L. plantarum* sa28k	Serum cholesterol reduction,	[34]
Sauerkraut (Kimchi)	*Lactobacillus acidophilus* KFRI342, *L. plantarum* NR74, *L. plantarum* C182, *L. plantarum* Ln4, *L. plantarum* EM, *L. plantarum* LPpnu, *L. plantarum* Ln1, *Leuconostoc mesenteroides* F27, *Leuconostoc mesenteroides* B1, *Leuconostoc mesenteroides* LMpnu, *Lactococcus lactis* KC24,	Anticancer effects, Serum cholesterol reduction, Antioxidant ability	[13, 27, 35–38]
Kimchi	*Weissella cibaria* JW15	Serum triglycerides reduction, feces ammonia emissions reduction	[39]
Fermented radish leaves (Dua-muoi)	*L. gaserri* HA4	Strengthening the digestive system, facilitate the digestion of food	[40]
Fermented green turnip (Japanese, Nozawana-zuke)	*L. fermentum* Nz8	Immunomodulation	[41]
Vegetable pickle (Tuscan pickles)	*L. plantarum* HY *L. plantarum* JR14	Antioxidant ability	[42, 43]
Fermented leek	*L. plantarum* LK8, *L. plantarum* IMDO 788, *L sakei* IMDO 1358, *Leuconostoc mesenteroides* IMDO 1347, *Weissella confusa* LK4	Antioxidant ability	[44]

(continued)

Table 1 (continued)

Fermented vegetable product	Isolated possible probiotic strains	Health-promoting effects	Reference/s
Fermented soybean (Tempeh)	*Rhizopus microsporus* var. *oligosporus* IFO 8631	Creation GABA	[24]
Korean soy sauce	*Bacillus subtilis* MKSK-E1, *B. subtilis* MKSKJ1	Antimicrobial activity	[25]
Fermented soybean (Japanese, Miso)	*Zygosaccharomyces sapae* strain I-6	Anti-inflammatory activity	[26]
Pickled vegetable (Iranian, Torshi)	*Bacillus amyloliquefaciens* 1020G *B. safensis* 437F, *B. atrophaeus* 1630F	Antioxidant activity, facilitate the digestion of food	[45]
Thai fermented stink bean (Sataw-Dong)	*L. plantarum* KJ03	Serum cholesterol reduction	[46]
Pickled cucumber (Korean, Jangajji)	*Saccharomyces cerevisiae* KU200278, *Saccharomyces cerevisiae* KU200281	DNA protection capacity against ROS	[47]
Jiangshui (Chinese fermented vegetables)	*Brevebacterium casei*, *Lactococcus raffinolactis*	Serum cholesterol reduction	[48]
Jiangshui	*Lactiplantibacillus plantarum*	Ant-pathogenic bacteria	[49]
Mixed fermented vegetable juices (purple cabbage, tomato and carrot)	*Lactobacillus plantarum*, *Saccharomyces cerevisiae*	Probiotic properties	[50]
Fermented vegetable	*Enterococcus hirae*	Increases immune responses, immune gene expression, protection to Aeromonas hydrophila infection	[51]
Pickled vegetables (in the Middle Eastern, African, and Asian sub-continent regions)	*Levilactobacillus namurensis*, *Lentilactobacillus buchneri*, *Lentilactobacillus parafarraginis*, *Pectobacterium carotovorum*, *Weissella confuse*, *Lactiplantibacillus pentosus*, *Leuconostoc carnosum*	Probiotic properties, resistance against clinically important antibiotics	[52]

(continued)

Table 1
(continued)

Fermented vegetable product	Isolated possible probiotic strains	Health-promoting effects	Reference/s
Fermented vegetable-fruit drink, combination of vegetable juice (broccoli, celery, asparagus, carrot extracts) and fruit juice (mulberry, grapes, passion fruit, blue berry, pineapple, apple, bayberry, cranberry, sugar cane, lemon extracts) in a mass ratio 1:1	*Saccharomyces cerevisiae*, *Streptococcus thermophiles* TCI125	Anti-oxidant and anti-aging activities	[53]
Fermented curly kale juice	*Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *L. sakei*, *L. coryniformis*	Ant-pathogenic bacteria	[54]

6. Probiotic culture (e.g., *Lactiplantibacillus plantarum* and *Lacticaseibacillus casei*) and others such as *Levilactobacillus brevis* e *Leuconostoc mesenteroides* (*see* **Note 1**).
7. Container or tank for washing and sanitizing vegetables.
8. System for heat treatment of brine and bleaching.
9. Glass packaging.
10. Incubator for fermentation.
11. Refrigeration system.

2.1.2 Methods

1. Select vegetable raw materials (*see* **Note 2**).
2. Wash the vegetable raw materials with drinking water.
3. Sanitize using chlorine-based products (*see* **Note 3**).
4. Rinse with fresh water 3–4 times to remove the sanitizer.
5. Cut the vegetables into cubes, slices, or rounds (*see* **Note 4**).
6. Blanch in boiling water for 2–4 min, followed by cooling in chilled water (*see* **Note 5**).
7. Prepare the brine with drinking water, add sucrose or glucose (1–3%, w/v) for the bacteria to grow, sodium chloride (3–5%, w/v) (*see* **Note 6**), and heat at 95 °C for 5 min in a water bath and cooled to microbial inoculation (*see* **Note 7**).
8. After cooling to 20–30 °C, add the probiotic culture and homogenize (*see* **Note 8**).
9. Pack the vegetables in a glass container and add the brine (*see* **Note 9**).

10. Ferment at the time and temperature (usually 20–30 °C) indicated for the probiotic culture used until it reaches a pH lower than 3.8 (*see* **Note 10**).
11. Store at refrigeration temperature.

2.1.3 Notes

1. Check the manufacturer's instructions for use in less than the recommended volume in the microbial culture envelope. More instructions are given in Chap. 5, in the **Notes** to Subheading 3.
2. Raw materials must be at the appropriate degree of maturation without physiological damage, mechanical injuries, or deterioration.
3. It is recommended to use sodium hypochlorite or sodium dichloroisocyanurate (150 ppm for 15 min). However, other sanitizers approved by a regulatory agency can be used.
4. Some vegetables can be used whole, for example, mini cucumbers and baby carrots.
5. The bleaching inactivates enzymes, reduces undesirable microorganisms, softens the texture, facilitates packaging, and contributes to preserving the color of vegetables.
6. In high concentrations of NaCl, the growth of *Leuconostoc mesenteroides* and other less salt-tolerant species is inhibited.
7. $CaCl_2$ can partially replace NaCl to avoid excessive softening if the objective is to obtain a crunchy texture during storage. Pasteurization of the brine before fermentation is necessary to ensure the safety and growth exclusivity of the inoculated starter culture.
8. It is recommended that the starter culture probiotic concentration be at least 7 log CFU/mL in brine to inhibit spoiling microbiota. In addition, after excessive acid accumulation, a reduction in the probiotic count can be observed during storage. Therefore, it is recommended to use *Lactiplantibacillus plantarum* and *Lacticaseibacillus casei* LA284 probiotics, which are acid resistant to pickle processing conditions, keeping their viability stable during storage [55]. *Lactobacillus acidophilus* has also been shown to be suitable for use in pickled vegetables. In addition, mixed cultures can be used, containing *Levilactobacillus brevis* and *Leuconostoc mesenteroides* ensured the most preferred end products regarding sensory properties [56]. The main species responsible for fermentation are homofermentative lactic acid bacteria, such as *Lactiplantibacillus plantarum*. During prolonged fermentation, lactic acid can be metabolized if spoilage microorganisms are present, triggering the production of spoilage by-products such as propionic acid, butyric acid, and CO_2. The CO_2 can cause economic losses, affecting the texture and bloater damage.

9. On an industrial scale, fermentation is usually carried out in tanks, and the product is packaged in smaller containers.
10. The fermentation time of pickles from vegetables fermented by *Lactiplantibacillus plantarum* is 20–30 °C for 72 h, but this may vary according to the microorganism used. When lactic acid bacteria are used, the pH of the product usually decreases rapidly in the first 24 h of fermentation due to the intense production of lactic acid by the probiotics.

3 Probiotic Sauerkraut

Sauerkraut is a product made from cabbage by fermentation of a mixed culture of bacteria, in the presence of sodium chloride and with a production of lactic acid. As it is a product fermented with lactic acid bacteria, it is a suitable substrate for the growth of probiotic bacteria. The main materials and processing steps for obtaining probiotic sauerkraut are described below:

3.1 Preparation of Probiotic Sauerkraut

3.1.1 Materials

1. White cabbage.
2. Drinking water.
3. Chlorine-based sanitizer (e.g., sodium hypochlorite or sodium dichloroisocyanurate).
4. Salt.
5. *Leuconostoc mesenteroides* and probiotic culture *Lactiplantibacillus plantarum* (*see* **Note 1**).
7. Container or tank for washing and sanitizing vegetables.
8. System for pressing and fermentation.
9. Glass or plastic packaging.
10. Incubator for fermentation.
11. Refrigeration system.

3.1.2 Methods

1. Select cabbage heads by removing the outer leaves and central stalk (*see* **Note 2**).
2. Wash the cabbage with drinking water.
3. Sanitize using chlorine-based products (*see* **Note 3**).
4. Rinse with fresh water 3–4 times to remove the sanitizer.
5. Cut the cabbage into thin (0.5–2 mm) and even slices.
6. Place the cabbage slices in the fermentation vessel or tank, and add sodium chloride (1–4%, w/w) (*see* **Note 4**).
7. Add a starter culture containing probiotic bacteria (*see* **Note 5**).
8. Pressing the product compresses it and facilitates the release of liquid during fermentation.

9. Keep fermentation vessels closed to promote anaerobic conditions (*see* **Note 6**).
10. Ferment at the time and temperature (usually 20–25 °C by 3–10 days) indicated for the probiotic culture used until it reaches at least 1.5% lactic acid and a pH lower than 3.8.
11. Pack the sauerkraut in previously sterilized glass or plastic jars.
12. Store at refrigeration temperature.

3.1.3 Notes

1. *Leuconostoc mesenteroids* present rapid adaptation in this substrate. Therefore, its growth is more significant at the beginning of fermentation. At the end of fermentation, the probiotic *Lactiplantibacillus plantarum* may predominate, which is more resistant to acidic conditions [57].
2. White cabbage heads without mechanical injuries or deterioration should be selected. Before processing, cabbage must be kept at room temperature for 36–48 h, so its leaves partially wilt.
3. It is recommended to use sodium hypochlorite or sodium dichloroisocyanurate (150 ppm for 15 min). However, other sanitizers approved by a regulatory agency can be used.
4. Salt promotes the removal of water from the plant cell, used by the fermenting microorganisms. In high concentrations of NaCl, the growth of *Leuconostoc mesenteroides* and other less salt-tolerant species is inhibited.
5. It is recommended that the starter culture probiotic concentration be at least 7 log CFU/g in product to inhibit spoiling microbiota.
6. Initially, the fermentation is aerobic, but anaerobic respiration begins due to the reduction of oxygen by the respiration of the vegetable and pressing. Therefore, preferably use a fermentation system that allows the CO_2 produced during fermentation to escape, reducing the internal pressure [57].

4 Probiotic Natto

Natto is a food made from the fermentation of cooked whole soybeans, popularly consumed by the Asian population. This product, which has sticky and viscous filaments, is obtained by fermentation of *Bacillus subtilis natto*. This microorganism has recently been investigated for its probiotic potential and was included in the list of probiotics of the Food and Drug Administration (FDA) [57]. The materials and processing steps for obtaining probiotic natto are described below:

4.1 Preparation of Probiotic Natto

4.1.1 Materials

1. Food-grade whole soybeans (*see* **Note 1**).
2. Drinking water.
3. Probiotic culture of *Bacillus subtilis natto* (*see* **Note 2**).
4. Container or tank for soaking soybean.
5. Pressure heat treatment system.
6. Polystyrene tray.
7. Room with temperature and humidity control for fermentation.

4.1.2 Methods

1. Select the soybeans, removing the impurities.
2. Wash dry whole soybeans and soak them in chilled water overnight. The volume of water is usually 2 to 3 times the volume of the bean.
3. Cook the soybeans (1.5 kg/cm^2) for 20 min (*see* **Note 3**).
4. Spray the probiotic culture (3–4 log UFC/g) and mix (*see* **Note 4**).
5. Transfer a portion to a polystyrene tray, in even layers of 4–5 centimeters, covering with a perforated plastic wrap to keep the moisture.
6. Ferment at 40 °C for 24 h (*see* **Note 5**).
7. Mature for 24 h under refrigeration and store at refrigeration temperature.

5 Notes

1. Preferred grains with tiny seeds and light hilum. Better sensory characteristics are obtained with soybean cultivars with lower protein content and higher carbohydrate and lipid content.
2. Commercial cultures can be used for direct vat set (DVS) inoculation or to prepare the inoculum on a laboratory scale. For laboratory scale, the strains can be inoculated in nutrient broth and fermented at 37 °C for 2 days, followed by centrifugation to obtain the pellet, which can be resuspended in sterile water to obtain the appropriate count of microorganisms [57].
3. Please note that the soybean hulls may come off during cooking, clogging the pressure release valve. Cooking can take place without pressure. However, the time will be much longer, and the beans must be cooked until a very soft texture is obtained.
4. If inoculation is carried out with vegetative cells, the soybeans must be previously cooled to 40 °C so as not to reduce the viability of *Bacillus subtilis natto*. However, suppose the inoculation is carried out with spores of *Bacillus subtilis* natto. In that

case, they can be inoculated with still-hot soybeans (80–90 °C) as these spores are resistant to heat, and thermal shock is important to activate them [57, 58].

5. Generally, the growth temperature is between 39 and 43 °C, and the ideal temperature for spore germination is 40 °C [58]. As the fermentation progresses, higher mucilage viscosity is observed, in addition to presenting an ammonia aroma.

References

1. Granato D, Barba FJ, Bursać Kovačević D, Lorenzo JM, Cruz AG et al (2020) Functional foods: product development, technological trends, efficacy testing, and safety. Ann Rev Food Sci Technol 11:93–118
2. Ansari F, Pourjafar H, Tabrizi A, Homayouni A (2020) The effects of probiotics and prebiotics on mental disorders: a review on depression, anxiety, Alzheimer, and autism spectrum disorders. Cur Pharm Biotechnol 21:555–565
3. Tripathi MK, Giri SK (2014) Probiotic functional foods: survival of probiotics during processing and storage. J Funct Food 9:225–241
4. Balthazar CF, Guimarães JF, Coutinho NM, Pimentel TC, Ranadheera CS et al (2022) The future of functional food: emerging technologies application on prebiotics, probiotics and postbiotics. Compr Rev Food Sci Food Saf 21:2560–2586
5. Pourjafar H, Ansari F, Sadeghi A, Samakkhah SA, Jafari SM (2022) Functional and health-promoting properties of probiotics' exopolysaccharides; isolation, characterization, and applications in the food industry. Crit Rev Food Sci Nutr:1–32
6. Aspri M, Papademas P, Tsaltas D (2020) Review on non-dairy probiotics and their use in non-dairy based products. Fermentation 6: 30
7. Min M, Bunt CR, Mason SL, Hussain MA (2019) Non-dairy probiotic food products: an emerging group of functional foods. Crit Rev Food Sci Nutr 59:2626–2641
8. Ansari F, Pourjafar H (2021) Nondairy foods as potential carriers of probiotic bacteria and Postbiotics. Probiotic bacteria and postbiotic metabolites: role in animal and human health: Springer pp 351–373
9. DiRienzo DB (2014) Effect of probiotics on biomarkers of cardiovascular disease: implications for heart-healthy diets. Nutr Rev 72:18–29
10. Fernandez MA, Marette A (2017) Potential health benefits of combining yogurt and fruits based on their probiotic and prebiotic properties. Adv Nutr 8:155S–164S
11. Ansari F, Pourjafar H (2022) Influence of fermentation and germination on food bioactives. Retention of bioactives in food processing: Springer pp 277–308
12. Szutowska J (2020) Functional properties of lactic acid bacteria in fermented fruit and vegetable juices: a systematic literature review. Eur Food Res Technol 246:357–372
13. Torres S, Verón H, Contreras L, Isla MI (2020) An overview of plant-autochthonous microorganisms and fermented vegetable foods. Food Sci Hum Wellness 9:112–123
14. de Melo Pereira GV, de Oliveira CB, Júnior AIM, Thomaz-Soccol V, Soccol CR (2018) How to select a probiotic? A review and update of methods and criteria. Biotechnol Adv 36: 2060–2076
15. Ayed L, M'hir S (2020) Hamdi M (2020) microbiological, biochemical, and functional aspects of fermented vegetable and fruit beverages. J Chem 2020:1
16. Patel A (2017) Probiotic fruit and vegetable juices-recent advances and future perspective. Int Food Res J 24:1850–1857
17. Martins EMF, Ramos AM, Vanzela ESL, Stringheta PC, de Oliveira Pinto CL et al (2013) Products of vegetable origin: a new alternative for the consumption of probiotic bacteria. Food Res Int 51:764–770
18. Ningrum DR, Budiwati TA, Nissa RC, Mawarda PC (2019) The production of freeze-dried probiotic from Nappa cabbage juice by lactic acid bacteria. Teknol Indones 41:33–41
19. Yoon KY, Woodams EE, Hang YD (2006) Production of probiotic cabbage juice by lactic acid bacteria. Bioresour Technol 97:1427–1430
20. Dzandu BA (2019) In vitro and in vivo (mouse model) investigation of the efficacy of probiotic bacteria lactobacillus Casei and lactobacillus Rhamnosus incorporated in tomato juice.

Dessertation 10.31390/gradschool_dissertations.5012

21. Yoon KY, Woodams EE, Hang YD (2005) Fermentation of beet juice by beneficial lactic acid bacteria. LWT-Food Sci Technol 38:73–75
22. Kun S, Rezessy-Szabó JM, Nguyen QD, Hoschke Á (2008) Changes of microbial population and some components in carrot juice during fermentation with selected Bifidobacterium strains. Process Biochem 43:816–821
23. Zhu W, Lyu F, Naumovski N, Ajlouni S, Ranadheera CS (2020) Functional efficacy of probiotic lactobacillus sanfranciscensis in apple, Orange and tomato juices with special reference to storage stability and in vitro gastrointestinal survival. Beverages 6:13
24. Aoki H, Uda I, Tagami K, Furuya Y, Endo Y et al (2003) The production of a new tempeh-like fermented soybean containing a high level of γ-aminobutyric acid by anaerobic incubation with Rhizopus. Biosci Biotechnol Biochem 67:1018–1023
25. Lee S, Lee J, Jin Y-I, Jeong J-C, Chang YH et al (2017) Probiotic characteristics of bacillus strains isolated from Korean traditional soy sauce. LWT Food Sci Technol 79:518–524
26. Okada Y, Tsuzuki Y, Furuhashi H, Sugihara N, Inaba K et al (2018) Tu1855-a novel probiotic yeast isolated from Japanese "Miso" exerts a therapeutic effect on DSS induced colitis possibly mediated by IL-10 producing Cd11C+ Dendritic cells. Gastroenterol 154:S-1039
27. Choi EA, Chang HC (2015) Cholesterol-lowering effects of a putative probiotic strain lactobacillus plantarum EM isolated from kimchi. LWT Food Sci Technol 62:210–217
28. Park MR, Oh S, Son SJ, Park D-J, Oh S et al (2015) Bacillus licheniformis isolated from traditional Korean food resources enhances the longevity of Caenorhabditis elegans through serotonin signaling. J Agri Food Chem 63:10227–10233
29. Banić M, Uroić K, Pavunc AL, Novak J, Zorić K et al (2018) Characterization of S-layer proteins of potential probiotic starter culture lactobacillus brevis SF9B isolated from sauerkraut. LWT Food Sci Technol 93:257–267
30. Beganović J, Kos B, Pavunc AL, Uroić K, Jokić M et al (2014) Traditionally produced sauerkraut as source of autochthonous functional starter cultures. Microbiol Res 169:623–632
31. Chiu HH, Tsai CC, Hsih HY, Tsen HY (2008) Screening from pickled vegetables the potential probiotic strains of lactic acid bacteria able to inhibit the salmonella invasion in mice. J Appl Microbiol 104:605–612
32. Yu Z, Zhang X, Li S, Li C, Li D et al (2013) Evaluation of probiotic properties of lactobacillus plantarum strains isolated from Chinese sauerkraut. World J Microbiol Biotechnol 29:489–498
33. Feng J, Liu P, Yang X, Zhao X (2015) Screening of immunomodulatory and adhesive lactobacillus with antagonistic activities against salmonella from fermented vegetables. World J Microbiol Biotechnol 31:1947–1954
34. Kusumawati N, Jenie B, Setyahadi S, Hariyadi R (2003) Selection indigenous lactic acid bacteria as probiotic strain with the ability to lower cholesterol. J Microbiol Indones 8:39–43
35. Jang HJ, Song MW, Lee N-K, Paik H-D (2018) Antioxidant effects of live and heat-killed probiotic lactobacillus plantarum Ln1 isolated from kimchi. J Food Sci Technol 55:3174–3180
36. Lee K-H, Bong Y-J, Lee HA, Kim H-Y, Park K-Y (2016) Probiotic effects of lactobacillus plantarum and Leuconostoc mesenteroides isolated from kimchi. J Korean Soc Food Sci Nutr 45:12–19
37. Lee KW, Shim JM, Park S-K, Heo H-J, Kim H-J et al (2016) Isolation of lactic acid bacteria with probiotic potentials from kimchi, traditional Korean fermented vegetable. LWT Food Sci Technol 71:130–137
38. Lee N-K, Han KJ, Son S-H, Eom SJ, Lee S-K et al (2015) Multifunctional effect of probiotic Lactococcus lactis KC24 isolated from kimchi. LWT Food Sci Technol 64:1036–1041
39. Sun HY, Kim KP, Bae CH, Choi AJ, Paik HD et al (2019) Evaluation of Weissella cibaria JW15 probiotic derived from fermented Korean vegetable product supplementation in diet on performance characteristics in adult beagle dog. Animals 9:581
40. Ho P, Wills M (2013) Identification of a potential probiotic strain, Lactobacillus gasseri HA4, from naturally fermented foods. https://sti.vista.gov.vn/file_DuLieu/dataTLKHCN//Vd1277-2015/2013/Vd1277-2015Q420130157.pdf
41. Kawahara T, Otani H (2006) Stimulatory effect of lactic acid bacteria from commercially available Nozawana-zuke pickle on cytokine expression by mouse spleen cells. Biosci Biotechnol Biochem 70:411–417
42. Liu T, Zhou K, Yin S, Liu S, Zhu Y et al (2019) Purification and characterization of an exopolysaccharide produced by lactobacillus plantarum HY isolated from home-made Sichuan pickle. Int J Biol Macromol 134:516–526
43. Yang M, Jiang R, Liu M, Chen S, He L et al (2017) Study of the probiotic properties of

lactic acid bacteria isolated from Chinese traditional fermented pickles. J Food Process Preserv 41:e12954

44. Yang J, Ji Y, Park H, Lee J, Park S et al (2014) Selection of functional lactic acid bacteria as starter cultures for the fermentation of Korean leek (Allium tuberosum Rottler ex Sprengel.). Int J Food Microbiol 191:164–171
45. Talebi S, Makhdoumi A, Bahreini M, Matin M, Moradi H (2018) Three novel bacillus strains from a traditional lacto-fermented pickle as potential probiotics. J Appl Microbiol 125: 888–896
46. Jampaphaeng K, Cocolin L, Maneerat S (2017) Selection and evaluation of functional characteristics of autochthonous lactic acid bacteria isolated from traditional fermented stinky bean (Sataw-Dong). Ann Microbiol 67:25–36
47. Hong J-Y, Son S-H, Hong S-P, Yi S-H, Kang SH et al (2019) Production of β-glucan, glutathione, and glutathione derivatives by probiotic Saccharomyces cerevisiae isolated from cucumber jangajji. LWT Food Sci Technol 100:114–118
48. Jun Z, Shuaishuai W, Lihua Z, Qilong M, Xi L et al (2018) Culture-dependent and-independent analysis of bacterial community structure in Jiangshui, a traditional Chinese fermented vegetable food. LWT Food Sci Technol 96: 244–250
49. Ou D, Ling N, Wang X, Zou Y, Dong J et al (2022) Safety assessment of one Lactiplantibacillus plantarum isolated from the traditional Chinese fermented vegetables—Jiangshui. Foods 11:2177
50. Yang F, Wang Y-p, Zhao H (2019) Quality enhancement of fermented vegetable juice by probiotic through fermented yam juice using Saccharomyces cerevisiae. Food Sci Technol 40:26–35
51. Hamid NH, Daud HM, Kayansamruaj P, Hassim HA, Yusoff MSM et al (2021) Short-and long-term probiotic effects of enterococcus hirae isolated from fermented vegetable wastes on the growth, immune responses, and disease resistance of hybrid catfish (Clarias gariepinus× Clarias macrocephalus). Fish Shellfish Immunol 114:1–19
52. Yasir M, Al-Zahrani IA, Bibi F, Abd El Ghany M, Azhar EI (2022) New insights of bacterial communities in fermented vegetables from shotgun metagenomics and identification of antibiotic resistance genes and probiotic bacteria. Food Res Int 157:111190
53. Chan L-P, Tseng Y-P, Liu C, Liang C-H (2021) Anti-oxidant and anti-aging activities of fermented vegetable-fruit drink. J Food Nutr Res 9:240–250
54. Szutowska J, Gwiazdowska D (2021) Probiotic potential of lactic acid bacteria obtained from fermented curly kale juice. Arch Microbiol 203: 975–988
55. Fan S, Breidt F, Price R, Pérez-Díaz I (2017) Survival and growth of probiotic lactic acid bacteria in refrigerated pickle products. J Food Sci 82(1):167–173
56. Di Cagno R, Filannino P, Gobbetti M (2015) Vegetable and fruit fermentation by lactic acid bacteria. In: Mozzi F, Raya RR, Vignolo GM (eds) Biotechnology of lactic acid bacteria: novel applications. John Wiley & Sons, New Jersey
57. Martin JGP, Lindner JD (eds) (2022) Microbiologia de alimentos fermentados. Bluncher, São Paulo
58. Kiuchi K, Watanabe S (2004) Industrialization of Japanese natto. In: Steinkraus KH (ed) Industrialization of indigenous fermented foods, 2nd edn. Marcel Dekker, New York

Chapter 9

Kombucha Production and Its Bioactive Compounds Analysis

Chun Zou, Yong-Quan Xu, Yi-Bin Huang, and Jun-Feng Yin

Abstract

Kombucha is a fermented beverage obtained from the fermentation of sweetened tea by a consortium of yeasts and bacteria. The beverage contains many bioactive compounds from tea extraction and microbial synthesis. In this chapter, we describe two preparation methods of Kombucha using a symbiotic culture of bacteria and yeast or a synthetic microbial community as a starter. Moreover, the determination of bioactive compounds, including organic acids, sugars, and catechins, has been introduced.

Key words Kombucha, Tea, Yeast, Bacteria, Bioactive compounds

1 Introduction

Kombucha is a functional beverage produced by the fermentation of sugared tea broth with a symbiotic culture of bacteria and yeast [1, 2]. There are many active compounds found in Kombucha [3, 4], such as tea polyphenols, vitamins (B1, B2, B6, B12, and C), organic acids (acetic acid, gluconic acid, glucuronic acid, among others), and D-saccharic-1,4-lactone acid. These active compounds provide Kombucha with potential benefits for human health [4, 5], including antioxidant, antimicrobial, and hepato-protective effects.

Kombucha is composed mainly of various acetic acid bacteria and yeasts, sometimes containing a little lactic acid [6]. The traditional culture method of Kombucha uses a symbiotic culture of bacteria and yeast (SCOBY) as a starter [7, 8], in which a cellulose film is formed. Still, it is difficult to control the microorganisms. Therefore, more and more researchers use a synthetic microbial community (SMC) as a starter for fine control [9, 10]. In this chapter, the preparation method of Kombucha using SCOBY or

Adriano Gomes da Cruz et al. (eds.), *Probiotic Foods and Beverages: Technologies and Protocols*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-3187-4_9,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2023

SMC has been introduced in detail. In addition, the determination of bioactive compounds, including organic acids, sugars, and catechins, has also been described.

2 Materials

2.1 Sugared Tea Broth

The composition of the sugared tea broth included the following materials (g/L) [11]: sucrose, 50–100; tea, 1.5–10.

2.2 Yeast Extract Peptone Dextrose (YPD) Broth

The composition of the YPD broth included the following materials (g/L) [12]: yeast extract, 10; peptone, 20; D-glucose, 20.

2.3 Hestrin-Schramm (HS) Medium

The composition of the HS medium included the following materials (g/L) [13]: glucose, 20; peptone, 5.0; yeast extract, 5.0; disodium phosphate (anhydrous), 2.7; and citric acid (monohydrate), 1.15; and final pH 5.0 ± 0.1.

2.4 De Man Rogosa Sharpe (MRS) Medium

The composition of the MRS medium included the following materials (g/L) [14]: glucose, 20; tryptone peptone, 10; beef extract, 8; yeast extract, 4; sodium acetate, 5; diammonium hydrogen citrate, 2; dipotassium hydrogen phosphate, 2; magnesium sulfate, 0.2; manganous sulfate, 0.05; Tween 80, 1; and final pH 6.5 ± 0.1.

3 Methods

3.1 Preparation Method of Kombucha

1. Preparing the sugared tea broth [15]: Mix 6 g of tea leaves with 100 g of sucrose in 1 L of boiling water and steep for 15 min (*see* **Note 1**). Then, filter the tea leaves and transfer the sugared tea broth into a sterilized glass jar. Cool the sugared tea broth to room temperature (*see* **Note 2**).
2. Inoculating starter cultures (SCOBY (a) or SMC (b)):
   (a) Use a SCOBY (*see* **Notes 3 and 4**) as a starter (Fig. 1). Inoculate 10 g of SCOBY into the glass jar bottle with the sugared tea broth, and seal with sterile gauze.
   (b) Use an SMC as a starter. Inoculate single colonies of yeast, acetic acid bacteria, and lactic acid bacteria into YPD broth, HS medium, and MRS medium (*see* **Note 2**), respectively, and culture at 30 °C with 160 rpm agitation for 24 h, at 30 °C with 160 rpm agitation for 48 h, and at 37 °C with no agitation for 24 h, respectively. Inoculate combinations of the three strains into the glass jar bottle with the sugared tea broth, and seal with sterile gauze.

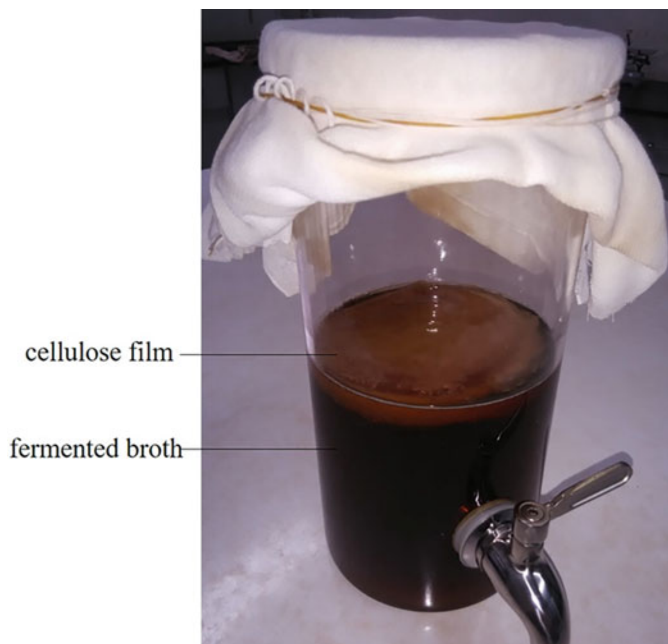

Fig. 1 Kombucha production using a SCOBY as starter

3. Culturing Kombucha: place the Kombucha cultures in an incubator (*see* **Note 5**) at 28 ± 2 °C, and let them stand for 1–2 weeks.
4. Post-fermentation treatment: after the fermentation process, remove the cellulose film (*see* **Note 4**), and collect the fermented broths (*see* **Note 6**).

3.2 Determination of Organic Acids

1. Mobile phase preparation.
   (a) Solvent A: Mix 970 mL of 0.1% phosphate solution and 30 mL of methanol in a 1 L glass bottle, then filter the mixture using a 0.45-μm microporous membrane.
   (b) Solvent B: 500 mL of 100% methanol (HPLC-grade).
2. Sample preparation.
   Centrifuge the fermented broths at 8000 rpm for 10 min, and collect the supernatants (*see* **Note 7**). Then, filter the supernatants using a 0.45-μm microporous membrane.
3. Instrument selection.
   HPLC equipped with a UV-DAD detector and an Agilent ZORBAX® SB-C18 column (4.6 × 150 mm, 5 μm).
4. LC settings [15].
   (a) Detection wavelength: 210 nm.
   (b) Column temperature: 40 °C.

(c) Injection volume: 10 μL.

(d) Flow rate: 1 mL/min.

(e) Gradient program: Maintain solvent A at 100% from 0 to 10 min, linearly ramp to 100% solvent B over 1 min, maintain solvent B at 100% for 5 min, linearly ramp to 100% solvent A over 1 min, and then maintain solvent A at 100% for 5 min.

3.3 Determination of Sugars

1. Mobile phase preparation.

   Solvent A: Mix 700 mL of acetonitrile and 300 mL of water in a 1 L glass bottle, and then filter the mixture using a 0.45-μm microporous membrane.

2. Sample preparation.

   Centrifuge the fermented broths at 8000 rpm for 10 min, and collect the supernatants. Then, filter the supernatants using a 0.45-μm microporous membrane.

3. Instrument selection.

   HPLC equipped with an evaporative light-scattering detector and a Waters XBridge™ Amdie column (4.6 × 150 mm, 5 μm).

4. LC settings [15].

   (a) Column temperature: 40 °C.

   (b) Injection volume: 10 μL.

   (c) Flow rate: 1 mL/min.

   (d) Elution mode: Isocratic mode.

   (e) Detector setting: Set drift tube temperature at 80—90 °C and nitrogen pressure at 350 kPa.

3.4 Determination of Catechins

1. Mobile phase preparation.

   (a) Solvent A: Add 90 mL acetonitrile, 20 mL acetic acid, and 2 mL EDTA-2Na solution (10 mg/mL) into a 1 L volumetric flask, add water to make a final volume of 1 L, and then filter the mixture using a 0.45-μm microporous membrane.

   (b) Solvent B: Add 800 mL acetonitrile, 20 mL acetic acid, and 2 mL EDTA-2Na solution (10 mg/mL) into a 1 L volumetric flask, add water to make a final volume of 1 L, and then filter the mixture using a 0.45-μm microporous membrane.

2. Sample preparation.

   Centrifuge the fermented broths at 8000 rpm for 10 min, and collect the supernatants. Then, filter the supernatants using a 0.45-μm microporous membrane.

3. Instrument selection.

   HPLC equipped with a UV-DAD detector and a Waters Symmetry C18 column (4.6 × 250 mm, 5 μm).

4. LC Settings [16].
   (a) Detection wavelength: 278 nm.
   (b) Column temperature: 35 °C.
   (c) Injection volume: 10 μL.
   (d) Flow rate: 1 mL/min.
   (e) Gradient program: Maintain solvent A at 100% from 0 to 10 min, linearly ramp to 68% solvent A and 32% solvent B over 15 min, maintain solvent A at 68% and solvent B at 32% for 10 min, linearly ramp to 100% solvent A over 1 min, and then maintain solvent A at 100% for 5 min.

4 Notes

1. The tea types used in Kombucha production are commonly black tea or green tea. Therefore, the time for extracting tea juice with boiling water should not be too long, which can avoid the flavor deterioration of tea juice.
2. All the media mentioned in "2 Materials" should be sterilized and cooled to room temperature before inoculation.
3. Starter cultures, SCOBY in particular, should not be contaminated by undesired microbes. If any mildew is found, the cultures should be discarded.
4. The SCOBY could be stored in a tea base liquid mixture. This can be kept at room temperature for up to 3 weeks, but for more extended storage, the SCOBY should be placed in the refrigerator at 4 °C.
5. Because vitamins in Kombucha are easily degraded under light, Kombucha should be cultivated in clean and dark conditions.
6. When the fermentation time is too long, the acidity of Kombucha might be too high to drink directly. Therefore, it can be appropriately diluted and mixed with sugar or honey before drinking.
7. When the organic acids of Kombucha are determined, the fermented broths should be diluted at a suitable multiple. It is due to the high concentration of organic acids in Kombucha.

References

1. Kapp JM, Sumner W (2019) Kombucha: a systematic review of the empirical evidence of human health benefit. Ann Epidemiol 30:66–70
2. Jayabalan R et al (2014) A review on kombucha tea—microbiology, composition, fermentation, beneficial effects, toxicity, and tea fungus. Compr Rev Food Sci Food Saf 13:538–550
3. Bortolomedi BM et al (2022) Bioactive compounds in kombucha: a review of substrate effect and fermentation conditions. Food Chem 385:1–16
4. Abaci N et al (2022) Kombucha – an ancient fermented beverage with desired bioactivities: a narrowed review. Food Chem X 14:1–10
5. Morales D (2020) Biological activities of kombucha beverages: the need of clinical evidence. Trends Food Sci Technol 105:323–333
6. Soares MG et al (2021) Technological aspects of kombucha, its applications and the symbiotic culture (SCOBY), and extraction of compounds of interest: a literature review. Trends Food Sci Technol 110:539–550
7. Harrison K, Curtin C (2021) Microbial composition of SCOBY starter cultures used by commercial kombucha brewers in North America. Microorganisms 9:1–21
8. Lee KR et al (2022) Kombucha fermentation using commercial kombucha pellicle and culture broth as starter. Food Sci Technol 42:1–7
9. Li RY et al (2022) Enhancing the proportion of gluconic acid with a microbial community reconstruction method to improve the taste quality of kombucha. LWT Food Sci Technol 155:1–8
10. Wang S et al (2020) Effect of synthetic microbial community on nutraceutical and sensory qualities of kombucha. Int J Food Sci Technol 55:3327–3333
11. Liang S et al (2021) Processing technologies for manufacturing tea beverages: from traditional to advanced hybrid processes. Trends Food Sci Technol 118:431–446
12. Nguyen NK et al (2015) Screening the optimal ratio of symbiosis between isolated yeast and acetic acid bacteria strain from traditional kombucha for high-level production of glucuronic acid. LWT Food Sci Technol 64:1149–1155
13. Han YH et al (2020) Ecofriendly green biosynthesis of bacterial cellulose by Komagataeibacter xylinus B2-1 using the shell extract of Sapindus mukorossi Gaertn. As culture medium. Cellulose 27:1255–1272
14. Zhang CC et al (2019) New selective media for isolation and enumeration of lactobacillus rhamnosus and Streptococcus thermophilus. J Food Meas Charact 13:1431–1439
15. Zou C et al (2021) Zijuan tea- based kombucha: physicochemical, sensorial, and antioxidant profile. Food Chem 363:1–8
16. Cao QQ et al (2019) Improving the taste of autumn green tea with tannase. Food Chem 277:432–437

Chapter 10

Probiotic Beer

Fernanda Meybom, Bárbara Mortl, and Alan Ambrosi

Abstract

The demand for functional beverages that provide health benefits to consumers has increased in recent years. In this sense, several studies investigate the addition of probiotics in beers. However, there are several challenges to overcome when adding probiotics to beer, such as the presence of alcohol and hop compounds that prevent the maintenance of a higher viable number of microorganisms. Thus, traditional beer production routes may not be recommended for this kind of product. Here, we provide a guideline on how to prepare a probiotic beer that can be used for researching new probiotic microorganisms and highlight essential points to be considered when developing probiotic beers.

Key words Functional beer, Functional beverage, Brewing, Yeast, *Levilactobacillus brevis*, *Saccharomyces cerevisiae*

1 Introduction

Beer is one of the oldest fermented beverages, dating back to the Neolithic age, and, nowadays, the most consumed alcoholic beverage in the world. Beer was considered food for several years, and sometimes, the only beverage safe to drink [1]. The beer industry has grown remarkably as time passed, especially during the Industrial Revolution, with technological improvements in equipment, ingredients, and implementation of scientific principles. In the last two decades, craft beers have experienced exponential growth, driven by consumers who seek unique drinking experiences. The current demand for health benefits or awareness about the importance of a healthy diet has driven the beer market to develop health-oriented beverages, like low/no alcohol beer, low-calorie beer, gluten-free beer, and functional beers [2].

Before it was known that microorganisms were responsible for transforming sugars (from grains) into ethanol, carbon dioxide, and a variety of volatile compounds, early beers were soured to some degree due to acidification by wild yeast and bacteria during

Adriano Gomes da Cruz et al. (eds.), *Probiotic Foods and Beverages: Technologies and Protocols*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-3187-4_10,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2023

spontaneous fermentation. Therefore, some traditional sour beers, which are intentionally acidified through wild lactic acid bacteria (LAB) and/or acetic acid bacteria (AAB), are considered a classic style of craft beer. Beer styles like Belgian Lambics and Flanders Red Ales represent some of the oldest commercial sour beers, which have recently seen a strong revival [3]. To avoid inconsistencies in aroma, flavor, quality, and long fermentation periods by using wild yeasts and wild LAB, pure or mixed commercial LAB cultures are preferred by brewers to control the brewing process, making a fast and reproducible biological acidification of wort [4].

Drinking unfiltered and unpasteurized beers rich in live probiotics is related to health benefits that regular beers might not provide. Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [5]. However, if the wild LAB and yeast involved in the spontaneous fermentation are not isolated and defined, and, if there is no evidence from well-designed clinical trials that suggests a possible health benefit, this undefined microbial consortium cannot be considered a probiotic [6].

Probiotic beer can be defined as a beverage obtained using probiotic microorganisms during the fermentation process [7]. The fermentation process can be conducted in one step, fermenting with one probiotic microorganism or co-fermenting using more than one microorganism [8, 9], or in two steps, fermenting with *Saccharomyces cerevisiae*, followed by fermentation with probiotic microorganisms [10]. However, producing probiotic beer is challenging. To guarantee high cell counts of live probiotics, the recommended minimum dosage is 9 Log colony-forming units (CFUs) per serving of product [5]. It is recommended to use yeasts as starter cultures to produce ethanol and carbon dioxide or to be cultured with probiotic microorganisms because probiotic LAB is incapable of fulfilling this primary purpose [11]. At the same time, the antimicrobial characteristic of hops, specifically iso-**α**-acid (17–55 ppm), can impair the growth and survival of probiotic LAB in beers [12]. In this case, using other hop derivatives such as hop essential oil is an alternative for allowing the growth of probiotic *Lactobacillus* spp. [11].

The *Bifidobacterium* and the strains of LAB—*Lactobacillus acidophilus, Lacticaseibacillus rhamnosus, Enterococcus,* and *Streptococcus*—are the most known probiotic microorganisms. The only commercial yeast used as a probiotic is *S. cerevisiae var. boulardii* [7]. Publications about probiotic beer are recent. Table 1 depicts the main probiotics being investigated to produce probiotic beers, including the beer style.

Table 1
Main probiotic microorganisms that are being investigated to produce probiotic beers

Probiotic	Beer style and process characteristics	Comments	References
L. acidophilus LA-5 and Bifidobacterium lactis BB-12	Low and alcohol-free beer Wort prepared in a commercial brewery	Starters: *Saccharomyces cerevisiae* 70,424 or *Saccharomyces rouxii* 2531 Probiotics were inoculated into the freshly made beer. After storage, cell counts were reduced, with greater losses in low-alcohol beer	[10]
Saccharomyces cerevisiae var. boulardii T1 compared with *Saccharomyces cerevisiae* Safbrew (T58)	No specific style Wort prepared with wheat and barley malts Each wort was fermented, maturated, and in-bottle refermented	After the process, cell counts were above 5 Log CFU/mL	[13]
Free and encapsulated *L. rhamnosus* GG (LGG) in alginate or alginate silica microcarriers	Pale lager Pasteurized Heineken	Encapsulation boosts the protection of the probiotic's cells during storage	[14]
S. cerevisiae var. boulardii in single and mixed cultures with *S. cerevisiae* 17 strains were isolated from natural matrices, then five were selected	No specific style Malted wort	*S. cerevisiae var. boulardii* (*Sb*) showed the ability to overcome stresses such as ethanol content, and it was dominant in almost all mixed cultures Mixed cultures increased the antioxidant capacity and total phenolic content	[15]
S. cerevisiae var. voulardii	Alcohol-free beer Synthetic medium containing dextrin, glucose, maltose, maltotriose, isomerized hop extract, and ethanol	Probiotic yeast was able to grow on a synthetic medium (highest specific growth rate on glucose) The effect of iso--acids on growth rate was not significant until 30 IBU The specific growth rate decreased at an ethanol level of 5% ABV	[16]

(continued)

Table 1 (continued)

Probiotic	Beer style and process characteristics	Comments	References
S. cerevisiae var. boulardii (CECT 1474) compared with *S. cerevisiae* (SF-04)	India Pale Ale Wort was made with a kit containing 100% hopped malt extract	Beer prepared with *S. cerevisiae var. boulardii* as a single yeast starter produced higher acidification, higher antioxidant activity, lower alcohol content, similar sensory attributes, and higher yeast viability after 45 days compared with the beer prepared with a commercial *S. cerevisiae*	[8]
L. paracasei L26 in coculture with *S. cerevisiae* S-04, and single cultures as control	Sour beer Sweet unhopped wort co-fermented with both microorganisms Late addition of isomerized hop extract	Unhopped wort was used as pre-culture and for co-fermentation. Lactic acid production and satisfactory growth of L26 were reported Storage in cold temperatures and live yeast enhanced the survival of L26 in hopped wort	[11]
Lactobacillus delbrueckii pure culture and *S. cerevisiae*	Pito—sour sorghum A portion of the wort was conducted in spontaneous lactic acid fermentation A portion was fermented in pito pure culture followed by S. cerevisiae fermentation	The sensory evaluation found no difference between pito brewed with starter cultures and the traditional pito. Application of the commercial starter cultures in fermenting pito extends shelf-life by 2 days over spontaneous pito	[17]
S. cerevisiae var. boulardii 17	Yeast extract-peptone-dextrose (YPD) agar broths with different concentrations of ethanol	*S. cerevisiae var. boulardii* had great resistance to alcohol and gastrointestinal conditions	[18]
S. cerevisiae var. boulardii CNCM I-745	Yeast extract-peptone-dextrose (YPD) agar broth Yeast was tested in YPD broth with 0 to 10% vol. of ethanol	*S. cerevisiae var. boulardii* CNCM I-745 was not able to grow at a concentration above 8% vol. at 28 °C and above 5% vol. at 37 °C Mathematical modeling of yeast stress resistance is a useful tool	[19]

Levilactobacillus brevis and *S. cerevisiae* SafAle S-3	Ale beer Pale malt extract and hopped wort After 2 weeks of fermenting, probiotic cells and dextrose were added to the beer	Free and immobilized cells remained viable at the end of the storage period (24 days) Cell counts, around 5 log CFU/mL after simulated gastric and intestinal fluids	[20]
Lacticaseibacillus paracasei subsp. *paracasei* DTA 81 (isolated from stools of infants 1 to 3 weeks old), *S. boulardii* 17, *S. cerevisiae* S-04	Wheat beer Sour beer Wheat beer was tested with hopped wort fermented with *S. boulardii* 17 Sour beer was fermented with *L. paracasei* DTA 81 and *S. cerevisiae* S-04 in an unhopped wort	*L. paracasei* DTA 81 survivability was compromised when dry hopping method was applied	[9]
43 wild yeast strains belonging to different genera such as *Lachancea*, *Kluyveromyces*, *Torulaspora*, *Metschnikowia*, *Kazachstania*, *Brettanomyces*, *Pichia*, *Candida*, *Hanseniaspora*, *Rhodotorula*, *Rodosporidobolus* and *Saccharomyces* Commercial *S. cerevisiae* US-05 and *S. boulardii* were used as control strains	Pilsner Pilsner with lentil Pilsner with chickpea flour	Pilsner with chickpea flour had an unpleasant aroma and taste. Pilsner with lentil had an effective fermentative character and pleasant aromatic notes. *S. cerevisiae*, *K. unispora*, and *L. thermotolerans* increased the main aromatic compounds in pilsner with lentils. These yeasts have the potential to produce a beer with high nutritional and functional characteristics	[7]
Saccharomyces cerevisiae var. boulardii and Commercial *S. cerevisiae W34/70* as first fermentation	Pilsen Wort from Heineken Brewery	According to the study, *S. boulardii* presented satisfactory tolerance to bile acid, pH, and ethanol Sensorial analysis showed good acceptance of the probiotic beer	[21]

Since beer is a complex liquid, obtained from a variety of raw materials and brewing routes, some variability can be expected in terms of characteristics, even when prepared at a laboratory scale. Thus, we present a protocol for the preparation of a probiotic beer based on a clear beer, with a refreshing taste, low alcohol content, clean lactic acidity, and a high level of carbonation, inspired by the sour beer styles (characterized by intentionally high acidity in beer) [22].

2 Materials

Probiotic beer is made from water, fermentable carbohydrates, hops, yeast, and probiotic microorganisms. The amount of each ingredient can be calculated by hand [23] or using free online calculators (for instance, BeerSmith2 and Brewer's Friend), considering the volume of beer and the values chosen for each vital characteristic of the sour beer style. In this protocol, we consider the preparation of 1 L of beer with the following characteristics: OG 1038 g/L, FG 1009 g/L, IBU 0, SRM 10, and ABV 3.7% v/v. The material can be acquired from local commerce (*see* **Note 1**). All the materials can be used at room temperature.

Water
1 L of fresh, filtered, and chlorine-free water (*see* **Note 2**). The recommended profile for this beer style is 50–60 ppm of calcium, 0–40 ppm alkalinity, 0–50 ppm sulfate, and 0–100 ppm chloride [24] (*see* **Note 3**).

Carbohydrate Source
0.3 kg of extra-light dry malt extract (DME) (*see* **Note 4**).

Hops
0.1 g of highly concentrated hop oil extracts (*see* **Note 5**).

Microorganisms
0.8 g of dry yeast *S. cerevisiae* for alcoholic fermentation. Keep the yeast refrigerated until using it (*see* **Note 6**).

1 mL of hydrated *L. brevis* for acid fermentation. This is the probiotic strain used in beer production (*see* **Note** 7). Keep the LAB refrigerated until using it.

Equipment
The main equipment required are presented in Table 2.

Table 2
Equipment required for preparing 1 L of probiotic beer

Item	Reason
2 units of 2 L glass autoclavable container/bottle	Wort preparation
1 unit of stove or heating plate	Wort heating
1 m of ¼″ food grade silicone hose	Transfer beer from the fermentation vessel
1 unit of a rubber stopper with an airlock	Releases the CO_2 from the fermentation bottle
Bowl	Fill with cold water to refrigerate the wort
Temperature chamber (0–25 °C)	Fermentation and maturation
Refractometer	Measure wort and beer densities (OG and FG)
4 units of 330 mL amber glass bottle	Packaging
4 units of metallic caps for the glass bottles	Packaging
Bottle capper	Packaging

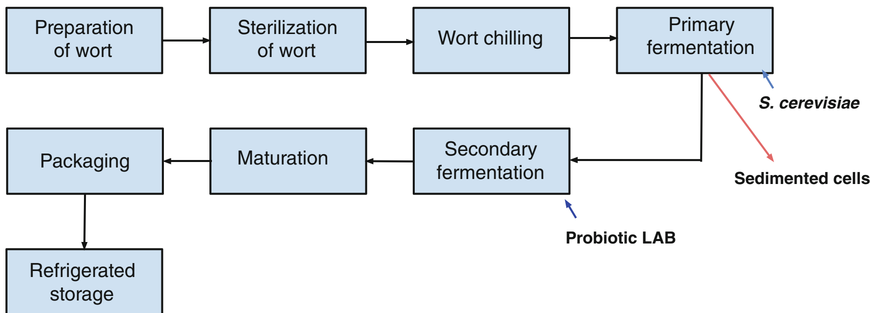

Fig. 1 Flowchart of the steps required to prepare the sour beer

3 Methods

The preparation of the sour beer follows the flowchart presented in Fig. 1 (*see* **Note 8**). Each step is described separately in this protocol.

3.1 Preparation of Wort

1. Prepare the wort using the 2 L glass container. Slowly, add the extra-light dry malt extract (DME) to 1.0 L of the previously prepared water. The mixture can be performed by using a magnetic stirrer.

3.2 Sterilization of Wort

1. After full solubilization of the DME, close the flask with aluminum foil or a cap (*see* **Note 9**).
2. Take the glass container to an autoclave. Set the temperature to 121 °C and let sterilize for 20 min [16].

3.3 Wort Chilling

1. Remove the container from the autoclave and let the temperature decrease to around 10 °C in the air (ambient condition).
2. Place the container in the water-cooling bath and let the temperature decrease to 18 °C (*see* **Note 10**). From here, be careful handling the wort, because it is susceptible to contamination.
3. After reaching the desired temperature, aerate the wort with 8–9 ppm oxygen [25]. The wort oxygenation process in the laboratory can be done by shaking the wort in the closed container for 10 min or until the minimum concentration is reached (*see* **Note 11**). Measurement of dissolved oxygen in wort can be performed by using an oximeter or other analytical chemical method.

3.4 Primary Fermentation

1. Remove the yeast from the refrigerator 1 h before using so that the cells are at a temperature close to the fermentation temperature. 0.8 g of the dry yeast *S. cerevisiae* is used for alcoholic fermentation (*see* **Note 12**).
2. Progressively sprinkle the dry yeast directly in the fermentation vessel on the surface of the wort, ensuring the yeast covers all the wort available to avoid clumps. Let the yeast be hydrated by the wort.
3. Close the container and shake it slowly to homogenize the yeast with the wort.
4. Remove the cap and attach the rubber stopper containing the airlock. This airlock relieves positive pressure due to the production of CO_2 during fermentation.
5. Place the container in the temperature chamber at 18 °C. The fermentation process continues until the final extract (FG) reaches 1009 g/L or it doesn't change in 48 h.
6. Cool the beer to 2 °C and keep it for 24 h to optimize the yeast sedimentation.
7. Slowly and carefully remove the supernatant liquid using the silicone hose and transfer it to a second fermentation vessel.

3.5 Secondary Fermentation

1. Remove the *L. brevis* from the refrigerator 1 h before using.
2. Prepare the beer for lactic fermentation (*see* **Note 13**) by heating it to 37 °C.
3. Shake the sachet with the microorganisms and inoculate 10 mL of the *L. brevis* directly into the fermentation vessel (*see* **Note 14**).
4. Cover the container and shake it to homogenize. Fermentation continues until a count of 9 Log CFUs is reached.

3.6 Maturation

1. After the second fermentation, cool the beer to 2 °C and keep it for 72 h for the maturation process (*see* **Note 15**).
2. To provide the probiotic sour beer with the flavor of hops, add 0.1 g of hop oil extracts directly to the liquid. After the addition, shake to homogenize the product (*see* **Note 16**).

3.7 Packaging

1. Pre-wash the glass bottles with mild soap and water and sanitize them with peracetic acid for 15 min (*see* **Note 17**). The sour beer should be bottled without going through a filtration and pasteurization process to ensure the permanence of living cells.
2. Pack the beer at a temperature of 2 °C and immediately cover it (*see* **Note 18**).

3.8 Refrigerated Storage

1. Keep the bottles at 5 °C to avoid the aging process and contain the fermentation [26]. The storage time should be as short as possible, a longer time will always have a negative influence on the quality and cellular viability of the probiotic beer.

4 Notes

1. Since the ingredients are from natural sources, they may have slight variations in their characteristics depending on the brand or even on the batch used. It is advisable to ask the supplier company for the specific analysis data for the batch of ingredients used.
2. Potable (tap) water can be treated by a series of filters; usually, a polypropylene filter (nominal pore size from 5–20μm) is followed by one or two steps of filtration through activated carbon to remove chlorine from water.
3. This water profile is a suggestion, based on the beer style. However, for research purposes, it is strongly recommended to use purified deionized water and analytical reagents to adjust the salt content and the required pH. Calcium sulfate, calcium chloride, magnesium sulfate, sodium bicarbonate, magnesium chloride, and sodium chloride can be used to adjust calcium (Ca^{+2}), magnesium (Mg^{+2}), bicarbonate (HCO_3^{-1}), sulfate (SO_4^{-2}), sodium (Na^{+1}), chloride (Cl^{-1}), and sulfate (SO_4^{-2}) ions [27].
4. Malted barley is the main carbohydrate source used for preparing beer, but other carbohydrate sources can also be used. The grain bill is calculated from the gravity units and color each carbohydrate source can offer to reach the desired beer OG and SRM [23].

However, to simplify laboratory studies, we suggest using the Dry Malt Extract. Using DME eliminates brewing steps (grain milling, mashing, and filtration).

5. Hops are used in beer production for impairing the aroma, flavor, and bitterness. In addition, they provide antimicrobial and antioxidant properties, which can impair probiotic growth [28]. Thus, replacing the addition of hops in the boiling stage with the use of concentrated hop extract in the maturation stage is an alternative to providing the beer with the hop aroma without harming the development of probiotics. We recommend using a concentrated hop extract with the aromatic character of grapefruit and tropical fruits.
6. Prefer using ale yeast with fast fermentation characteristics and the ability to form a compact sediment at the end of fermentation, which helps to improve the clarity of the beer.
7. There is a wide variety of probiotic species that can be used, such as *L. acidophilus, L. helveticus, L. delbrueckii subsp. bulgaricus,* and *L. paracasei*.
8. This is a proposed procedure, intended to facilitate the preparation of the sour beer at a laboratory scale and reach the desired characteristics, essential for research purposes.
9. If using a screw cap, be careful not to close the flask completely when autoclaving.
10. Ice can be used in the water bath to help decrease the temperature. The wort temperature should be just right for the specific yeast strain. For the sour beer style, the first fermentation with *S. cerevisiae* S-04 is performed at 18 °C.
11. In breweries, the wort is oxygenated using a medical oxygen cylinder with a flow meter. This equipment indicates the volume of air that dissolves in the liquid with scales from 0–15 L/min.
12. The dosage recommended in this protocol is 80 g/hL for fermenting at a temperature from 18 to 26 °C [29]. If using another yeast, follow the manufacturer's recommendation for dosage and fermentation temperature.
13. For producing probiotic sour beer, the acidification process is conducted after the primary fermentation (alcoholic fermentation) rather than the kettle sour process (common for traditional sour beer). This procedure avoids removing probiotic bacteria with the spent yeast from primary fermentation. Then, beer souring occurs after primary fermentation and removal of yeast [30].
14. The recommended concentration of the probiotic is 200 mL for 20 L of beer [31]. If using another variety of probiotic species, follow the manufacturer's recommendation for dosage and fermentation temperature.

15. During maturation, beer is saturated with carbon dioxide, and part of the turbidity-forming components of the beer settles (clarification) [25].
16. We recommend testing a dosage of 10 g/hL (range 5–40 g/hL) [32]. If using another variety of hop oil, follow the manufacturer's recommendation for dosage.
17. Follow the manufacturer's recommendation regarding the dilution of the peracetic acid.
18. There will probably be an accumulation of carbon dioxide and an increase in pressure in the bottle due to the production of carbon dioxide by the heterofermentation of *Lactobacillus* spp. [30]. In this case, the glass bottle is the most suitable because it supports a higher pressure compared to the plastic bottle. A low temperature of the beer in the bottle increases the solubility of carbon dioxide, it is recommended to pack at a temperature of 2 °C [26].

Acknowledgments

The authors are grateful to the Research and Innovation Support Foundation of Santa Catarina State (FAPESC), to CNPq (National Council for Scientific and Technological Development), and CAPES (Coordination of Improvement of Higher Education Personnel).

References

1. Standage T (2006) A history of the world in six glasses. Walker & Company, New York
2. Donadini G, Fumi MD, Kordialik-bogacka E, Maggi L, Lambri M, Sckokai P (2016) Consumer interest in specialty beers in three European markets. Food Res Int 85:301–314
3. Verachert H, Derdelinckx G (2014) Belgian acidic beers daily reminiscences of the past. Cerevisia 38:121–128
4. Peyer LC, Zarnkow M, Jacob F, De Schutter DP, Arendt EK (2017) Sour Brewing: impact of *Lactobacillus amylovorus* FST2.11 on technological and quality attributes of acid beers. J Am Soc Brew Chem:207–216. https://doi.org/10.1094/ASBCJ-2017-3861-01
5. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC, Sanders ME (2014) The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol 11:506–514
6. Sanders ME, Merenstein D, Merrifield CA, Hutkins R (2018) Probiotics for human use. Nutr Bull 43:212–225
7. Canonico L, Zannini E, Ciani M, Comitini F (2021) Assessment of non-conventional yeasts with potential probiotics for protein-fortified craft beer production. LWT Food Sci Technol 145:111361
8. Mulero-Cerezo J, Briz-Redón A, Serrano-Aroca A (2019) *Saccharomyces cerevisiae* Var. Boulardii: valuable probiotic starter for craft beer production. Appl Sci 9:3250
9. Silva LC, Lago HS, Rocha MOT, Oliveira VS, Melo RL, Sutz ETG, Paula BP, Martins JFP, Luchese RH, Guerra AF, Rodrigues P (2021) Craft beers fermented by potential probiotic yeast or *Lacticaseibacilli* strains promote antidepressant-like behavior in Swiss Webster mice. Probiotics Antimicrob Proteins 13:698–708

10. Sohrabvandi S, Razavi SH, Mousavi SM, Mortazavian AM (2010) Viability of probiotic bacteria in low alcohol – and non-alcoholic beer during refrigerated storage. Philippine Agricultural Scientist – FAO/AGRIS, Rome
11. Chan MZA, Toh M, Liu SQ (2021) Beer with probiotics and prebiotics in probiotics and prebiotics in foods – challenges, innovations, and advances. Elsevier, Amsterdam
12. Vriesekoop F, Krahl M, Hucker B, Menz G (2012) 125th anniversary review: bacteria in brewing: the good, the bad and the ugly. The Institute of Brewing & Distilling 118:335–345
13. Iniesta MF, Rubio SM, Roda RMC, Rodríguez TA (2016) Cerveza obtenida mediante fermentación con *Saccharomyces boulardii*. Patent ES2583178B1
14. Haffner FB, Pasc A (2018) Freeze-dried alginate-silica microparticles as carriers of probiotic bacteria in apple juice and beer. LWT Food Sci Technol 91:175–179
15. Capece A, Romaniello R, Pietrafesa A, Siesto G, Pietrafesa R, Zambuto M, Romano P (2018) Use of *Saccharomyces cerevisiae* var. *boulardii* in co-fermentations with S.cerevisiae for the production of craft beers with potential healthy value-added. Int J Food Microbiol 284:22–30
16. Senkarcinova B, Dias IAG, Nespor J, Branyik T (2019) Probiotic alcohol-free beer made with *Saccharomyces cerevisiae* var. *boulardii*. LWT Food Sci Technol 100:362–367
17. Djameh C, Ellis WO, Oduro I, Saalia FK, Blay YM, Komlaga GA (2019) West African sorghum beer fermented with *Lactobacillus delbrueckii* and *Saccharomyces cerevisiae*: shelf-life and consumer acceptance. J Inst Brew 125: 333–341
18. de Paula BP, Chávez DWH, Lemos Junior WJF, Guerra AF, Corrêa MFD, Pereira KS, Coelho MAZ (2019) Growth parameters and survivability of *Saccharomyces boulardii* for probiotic alcoholic beverages development. Front Microbiol. https://doi.org/10.3389/fmicb.2019.02092
19. Ramirez-Cota GY, López-Villegas EO, Jiménez-Aparicio AR, Hernández-Sánchez H (2021) Modeling the ethanol tolerance of the probiotic yeast *Saccharomyces cerevisiae* var. *boulardii* CNCM I-745 for its possible use in functional beer. Probiotics Antimicrob Proteins 13:187–194
20. Calumba KF, Reyes V, Bonilla F, Villasmil E, Sathivel S (2021) Ale beer containing free and immobilized *Lactobacillus brevis*, a potential delivery system for probiotics. Food Prod Process Nutr 3:8
21. Reitenbach AF, Iwassa IJ, Barros BCB (2021) Production of functional beer with the addition of probiotic: *Saccharomyces boulardii*. Res Soc Dev 10:2
22. BJCP. 28 American Wild Ale. https://www.bjcp.org/style/2021/28
23. Daniels R (2000) Designing great beers – the ultimate guide to brewing classic beer styles. Brewers Publications, Boulder, 568 p
24. Palmer J, Kaminski C (2013) Water, a comprehensive guide for brewer. Brewers Publications, Boulder
25. Kunze W (1999) Technology brewing and malting, 2nd edn. VLB Berlin, Berlin
26. Ziegler H (ed) (2009) Handbook of brewing: processes, technology, markets. Wiley, Weinheim
27. Palmer J (2006) How to brew – everything you need to know to brew beer right the first time, 3rd edn. Brewers Publications, Boulder, 248 p
28. Pires E, Brányik T (2015) An overview of the brewing process. In: Biochemistry of beer fermentation, 1st edn. Springer, Cham, 80 p
29. SafAle™ S-04. Available at: https://fermentis.com/en/product/safale-s-04/. Accessed on 25 Aug 2022
30. Chan MZAC, Chua JY, Toh M, Liu S-Q (2019) Survival of probiotic strain *Lactobacillus paracasei* L26 during co-fermentation with *S. cerevisiae* for the development of a novel beer beverage. Food Microbiol 82:541–550
31. *Lactobacillus brevis*. Available at: https://levteck.com.br/produto/lactobacillus-brevis/. Accessed on 25 Aug 2022
32. USING HOP OILS. Available at: https://nzhopoils.com/?page_id=91. Accessed on 25 Aug 2022

Chapter 11

Probiotic Fermented Sausage

Claudio M. E. Malaghini, Ana Paula Zapelini de Melo, and Silvani Verruck

Abstract

Many changes and innovations in fermented meat products have occurred over time, especially when it comes to health-promoting products. Some studies have been conducted with the aim of developing probiotic meat products that can improve the functionality of the gut microbiota. However, the technological challenges faced during the production of fermented meat sausages make it difficult to apply probiotics in these food matrices. For probiotics to deliver the expected health outcomes for consumers, they need to grow in the products and at the end the viable cell count must be sufficient for the microorganisms to reach the consumer's gut. Therefore, in this chapter we describe a protocol for probiotic Friolano-type sausage. Furthermore, the possible sources of defects in the production of probiotic salami and the best alternatives to overcome them are presented.

Key words Functional foods, Health-promoting compounds, Probiotic salami, Probiotic *Friolano salami*, Probiotic meat product

1 Introduction

Meat sausages are products elaborated with meat or edible organs, seasoned and smoked, and can be cured, cooked or dried, wrapped in tripe, bladder or other animal membrane properly cleaned [1]. Fermented sausages are products that undergo a rapid initial fermentation followed by partial dehydration and may or may not be smoked. They are meat products consisting mainly of pork or beef, but can be produced with other meat types, in addition to pork fat, salt, sugar, curing agent, spices and starter cultures. They do not require refrigeration and have great stability compared to other meat products [2].

Friolano Salami is a kind of fermented sausage made exclusively from pork and lard, ground to a medium particle size of 6–9 mm and with the addition of the other ingredients required. It has an irregular cylindrical shape (defined by the shape of the natural wrap) with length ranging from 15 to 130 cm. Its weight ranges from 0.2 to 4.5 kg and presents non-elastic consistency, compact mass,

Adriano Gomes da Cruz et al. (eds.), *Probiotic Foods and Beverages: Technologies and Protocols*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-3187-4_11,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2023

delicate aroma, sweet and delicate flavor, and ruby red color without spots. It is a cured product, which can go through the smoking process, being fermented, matured, and dried [2].

In the world of fermented meat sausages many changes and innovations have taken place over time, especially when it comes to products that are beneficial to health, since the demand for these foods has become a priority for many consumers. With this in mind, studies have been conducted with the addition of probiotics in meat sausages [3].

Probiotics are live microorganisms that when properly added to products present benefits to the consumer's health, with specific effects and functional properties [4]. In addition, they contribute to the balance of intestinal microflora, helping the intestinal transit and facilitating digestion, relieve the symptoms of lactose intolerance, prevent colon cancer, reduce cholesterol and blood pressure, stimulate the immune system, produce B-complex vitamins, digestive and protective enzymes, protect against pathogenic microorganisms and control inflammatory vessel diseases [4–6].

However, for them to present the expected results they need to grow in the products, and at the end of the shelf life the viable cell count should be enough for the microorganisms to reach the consumer's intestine, which makes their application in fermented sausages difficult, due to their high acidity and salt content, and lower water activity (a_w) [4]. Therefore, in some studies probiotics are added microencapsulated in foods, which ensures the viability of the probiotic microorganisms during the process and in the final product [6]. Therefore, this chapter is directed towards the design of a probiotic *Friolano Salami* protocol. Furthermore, the possible sources of defects in meat sausages and the best alternatives to overcome them are presented.

2 Materials

1. Pork shank meat.
2. Back fat (lard).
3. Sodium chloride.
4. Curing salts (sodium nitrate).
5. Sugar.
6. Garlic powder.
7. Chili powder.
8. Probiotic culture (Table 1).
9. Starter culture (Table 2).
10. Collagen wrap with 50 a 60 mm.

Table 1
Probiotic or potentially probiotic cultures in meat products and the main results found in the studies

Product	Probiotic or potential probiotic used	Main results	References
Fresh pork sausage	*L. sakei* BAS0117 isolated from Brazilian fermented meat products (Italian salami, Calabrian sausage, ham, and mortadella)	The strains added allowed desirable characteristics to the product during storage	[7]
Italian salami sausage	*L. acidophilus*, *Bifidobacterium lactis* (potentially probiotic)	The addition of probiotic cultures produced sausages with good physical-chemical, microbiological, and sensory properties	[8]
Fermented pork sausage and loin	*L. rhamnosus* LOCK900 (probiotic strain)	Many lactic acid-producing bacteria, including 90% *L. rhamnosus,* were found during all stages of the meat process The added probiotics inhibited lipid oxidation in loins and pork sausage	[9]
Sausage and pork neck fermented and dry-cured	Pure cultures of probiotic strains: *Bifidobacterium animalis* subsp. *lactis* BB-12 (strain deposit number: DSM15954), *L. rhamnosus* LOCK900 (strain deposit number: CP005484), *L. acidophilus* Bauer (potentially probiotic)	The three starter strains could be applied to smoked meat products. However, the culture *L. acidophilus* Bauer did not allow lipid oxidation and discoloration of the products	[10]
Fermented sausage	Isolated from human intestinal tracts: *L. acidophilus* FERM P-15119, *L. rhamnosus* FERM P-15120, *L. paracasei subsp. paracasei* FERM P-15121 Commercial starter culture: *L. sake* (Chr. Hansen's)	The three strains examined could inhibit the growth of *S. aureus* and enterotoxins during the sausage fermentation process at different temperature variations	[11]
Fermented sausage	Probiotic lactobacilli strain isolated from infants' feces: *L. casei/paracasei CTC1677*, *L. casei/paracasei CTC1678*, *L. rhamnosus CTC1679* (confirmed probiotic lactobacilli) Commercial probiotic strains: *L. plantarum 299v*, *L. rhamnosus GG*, *L. casei Shirota*	It was observed that the *L. rhamnosus* remained viable at high levels (10^8 CFU/g) and survived the passage in TGI during sausage consumption	[12]

(continued)

Table 1 (continued)

Product	Probiotic or potential probiotic used	Main results	References
Fermented sausage	LAB isolated from infants qualified as potential probiotics: *L. gasseri CTC1700*, *L. gasseri CTC1704*, *L. fermentum CTC1693* Potential probiotic strains with proved ability, isolated from infant feces: *L. casei/paracasei CTC1677*, *L. casei/paracasei CTC1678*, *L. rhamnosus CTC1679*	*L. rhamnosus CTC1679* was the only strain capable of mastering both repetitions A putative probiotic effect can be achieved by eating 10 g/day of fuet with CTC1679	[13]
Fermented sausage	Probiotic strain: *E. faecium ATCC 8459*	*E. faecium* was efficient as a starter for producing fermented sausage with resistance to curing salts and sodium chloride and maintained its viability during the ripening process	[14]
Fermented sausage	*E. faecium CRL183* (potential probiotic)	It demonstrated a positive influence of sausage fermented with *E. faecium* CRL183 on microbial diversity	[15]
Fermented sausage	*L. rhamnosus* CTC1679 (potential probiotic)	*L. rhamnosus CTC1679* used as a probiotic starter culture produced safe, nutritionally enhanced fermented sausages The strain showed the ability to act as probiotic starter cultures remaining viable at high levels (108 CFU/g) in ripened fuets and surviving the passage through the human GIT during the consumption of the sausages	[16]
Fermented sausage	*Commercial probiotic strain: L. sakei* (potential probiotic)	The strains presented technological characteristics expected for application in sausage maturation processes as a starter culture	[17]

Raw fermented sausage	*L. casei* LOCK 0900 isolated from feces of healthy infants (potential probiotic)	Raw fermented sausages with probiotic strain *L. casei* LOCK 0900 showed good microbiological quality. The environment of raw fermented sausages is suitable for the growth and survival of the probiotic strain *L. casei* LOCK 0900	[18]
Fermented lamb sausage	*Commercial probiotic strain: L. acidophilus* CCDM 476, *Bifidobacterium animalis* 241a (potential probiotic)	The number of *Lactobacillus* (10^7 CFU/g) and *Bifidobacterium* (10^3 CFU/g) in the final product did not alter its technological properties. Despite this, there were problems in using *Bifidobacterium* as a starter because of its low concentration after fermentation and absence after 60 days of storage	[19]
Norwegian fermented sausage, Swedish fermented sausage, and Norwegian cured ham	Potential probiotic cultures isolated from fermented meat: *L. sakei* MF1295, *L. sakei* MF1296, *L. farciminis* MF1288, *L. plantarum/pentosus* MF1290, *L. plantarum/pentosus* MF1299, *L. plantarum* MF1291, *L. plantarum* MF1297, *L. pentosus* MF1300, *L. alimentarius* MF1297	The strains met all probiotic criteria and proved to be rapidly producing lactic acid, demonstrating the successful application of the selected strains as starter cultures for Scandinavian-type fermented sausages	[20]
Dry fermented sausage	*L. rhamnosus* GG (probiotic strain), *L. rhamnosus* E-97800 (potential probiotic), *L. rhamnosus* LC-705 (potential probiotic) Commercial strains: *Pediococcus pentosaceus*	*L. rhamnosus* E-97800 showed the fastest growth and acidification rate. Therefore, *L. rhamnosus* GG and *L. rhamnosus* E-97800 were considered tasty as the sausages fermented by the control	[21]
Dry fermented sausage	Commercial probiotic strains documented: *L. rhamnosus* R0011, *L. helveticus* R0052, *L. rhamnosus* Lr- 32, *L. paracasei* Lpc-37, *L. casei* Shirota, *L. reuteri* DSM17938, *L. reuteri* DSM17918, *Enterococcus faecium* MXVK29	The evaluated strains demonstrated different technological capacities in the other conditions in which the tests were performed *L. rhamnosus* Lr-32, *L. rhamnosus* R0011, *L. paracasei* Lpc-37, *E. faecium* MXVK29, and *L. casei* Shirota strains are the primary candidates to be used as sausages starters culture	[22]

(continued)

Table 1
(continued)

Product	Probiotic or potential probiotic used	Main results	References
Iberian dry fermented sausage	Probiotic culture: *L. fermentum* HL57, *Pediococcus acidilactici* SP979	Inoculation with *L. fermentum* HL57 increased the amount of acetic acid and lipid degradation products, such as malonaldehyde in Iberian dry fermented sausages, resulting in a negative impact on relevant sensory parameters related to color and flavor. On the contrary, *P. acidilactici* SP979 did not remarkably modify the physical–chemical parameters or sensory quality of Iberian dry-fermented sausages	[23]
Tunisian dry fermented sausage	Autochthonous strains isolated from a Tunisian traditional salted meat "kadid": *L. plantarum*, *S. xylosus* (not confirmed probiotic)	The use of bacterial strains can inhibit the growth of Gram-negative bacteria and may improve the sensory properties of sausage due to nitrate reductase and protease activity of *the S. xylosus* strain and the acidifying activity of the *L. plantarum* strain	[24]
Harbin dry sausage	Potential probiotics: *P. pentosaceus* R1, *L. brevis* R4, *L. curvatus* R5, *L. fermentum* R6 Confirmed probiotics from fermented dairy products (used for comparison with the isolated LAB): *L. acidophilus* AD1, *L. plantarum* KLDS1.0391, *L. curvatus* KLDS1.0505, *L. sake* AS1, *L. pentosaceus* KLDS1.0412, *L. fermentum* KLDS1.0709	Except for *L. curvatus* R5, all strains isolated from Harbin dry sausages supported passage in the gastrointestinal tract (GIT). Different components of the strains have different modes of antioxidant action. LAB isolated from Harbin dry sausages has strong probiotic properties and can be used as potential probiotics for food processing	[25]
Sucuk-type dry sausage	Twenty-three probiotic *L. plantarum* strains producing the conjugated linoleic acid (CLA) were screened: *L. plantarum* LMG 11405 and *L. plantarum* LMG 23521 were selected from the catalog of	*L. plantarum* AA1-2 and *L. plantarum* AB20-961 were identified as potential strains for CLA production. The strain of *L. plantarum* AB20-961 can be used to produce sucuk by an increase in the amount of linoleic acid of the	[26]

	BCCM/LMG (Belgian Coordinated Collections of Microorganisms/Laboratory of Microbiology) at the University of Ghent Twenty one *L. plantarum* strains (AA1-2, AA13-59, AA17-73, AB6-25, AB7-35, AB16-65, AB20-961, AC3-27, AC10-40, AC18-82, AC21-101, AC21-1031, AC18-88, AC3-10, AC3-14, AK4-11, AK6-27, AK6-28, AK8-31B, BC18-81, BK10-48) isolated from human sources in Suleyman Demirel University	product, without alteration on the product's final characteristics Production conditions such as temperature and pH were probably the most limiting factors for linoleic acid production	
Salami	Commercial probiotics: *L. plantarum* 299v, *L. plantarum* DSM 9843, *L. rhamnosus* LbGG or ATCC 53103, *L. casei* Shirota YIT 9029, *L. reuteri* DSM 17938, *L. casei* ATCC 393	The microbiological counts were different according to the type of starter strain used. *L. plantarum* 299v kept a concentration higher than 106 CFU g^{-1}, level of probiotic bacteria recommended at the time of consumption to exert a beneficial effect in humans The experimental salami proved to be safe since coagulase-positive coliforms and *Staphylococci* were not detected in the salami at the moment of consumption and after more than 1 month of storage at a cooling temperature	[27]
Nostrano salami	Bacterial strains: *L. lactis ssp. lactis* strain 340, *L. lactis ssp. lactis* strain 16, *L. casei ssp. casei* strain 208, *E. faecium* UBEF-41	These strains were selected for its ability to grow under low temperatures and modulate the aroma by converting amino acids and fatty acids, making it possible to produce fermented matured at low temperature without adding nitrates and nitrites, resulting in a potentially safer product with no adverse effect on the quality of Italian salami	[28]

(continued)

Table 1
(continued)

Product	Probiotic or potential probiotic used	Main results	References
Italian-type salami	Probiotic cultures: *L. casei* LC 01, *L. paracasei* ssp. *paracasei* ATCC 10746/CCT 0566, *L. rhamnosus* ATCC 7469/CCT 6645	The addition of these strains to the sausages caused a reduction in the development of *S. xylosus*. On the other hand, probiotic cultures did not interfere in the growth of *P. pentosaceus*, which, for most evaluation periods, showed better development when together with *Lacticaseibacillus*	[29]

Adapted from ref. [3]

Table 2
Main starter cultures used in meat sausages

Starter culture	Strain
Acid lactic bacteria	*Latilactobacillus sakei* *Latilactobacillus curvatus* *Lactiplantibacillus plantarum* *Lacticaseibacillus rhamnosus*
Pediococus	*Pediococcus acidilactici* *Pediococcus pentosaceus*
Staphylococcus and *Kocuria*	*Staphylococcus xylosus* *Staphylococcus carnosus* *Staphylococcus equorum* *Kocuria varians*
Micrococcaceae	*Micrococcaceae candidus* *Micrococcaceae aquatilis*

2.1 Equipment

1. Meat grinder with 6–9 mm disc.
2. Lard cuber.
3. Mixer.
4. Maturation chamber.
5. Drying chamber.

3 Methods

The protocol for making a probiotic *Friolano Salami* is illustrated in Fig. 1.

3.1 Probiotic Cultures

1. Use direct vat set (DVS) or direct vat inoculation (DVI) cultures that allow the addition of probiotic strains directly into the food matrix. See Chapter 13 for the main suppliers of DVS probiotic strains (Table 1).

3.2 Friolano Salami Manufacture

1. Grind the raw pork meat (85% of the raw material) with a 6–9 mm disc at a temperature of 4–7 °C and then transfer it to the blender.
2. Cut the bacon (15% of the raw material) in an incubator at 0 °C into cubes of no more than 1 cm. Add the lard to the meat in the blender.
3. Add 2.5% sodium chloride, 0.3% sugar, 0.03% chili powder, 0.3% garlic powder, and 0.015% sodium nitrate over the meat in the blender.

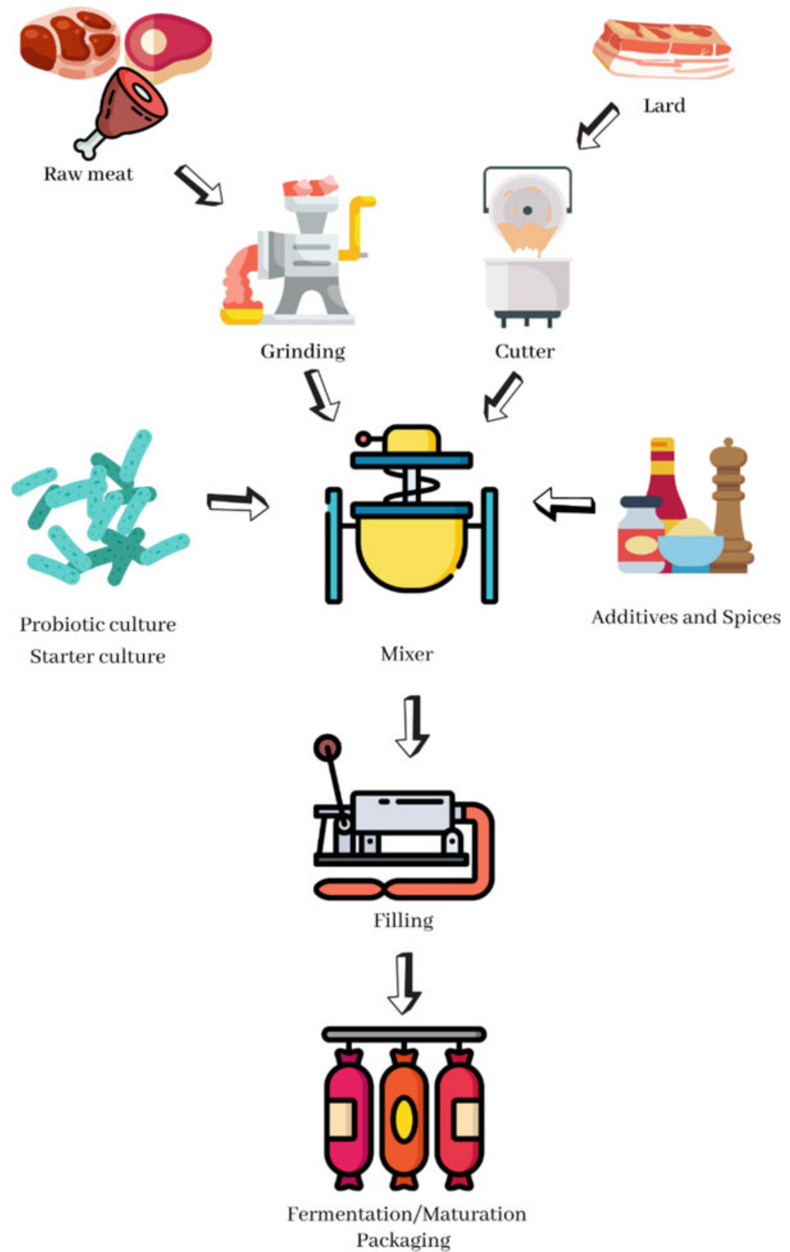

Fig. 1 Steps for the elaboration of a probiotic *Friolano Salami*

4. Mix the ground meat, minced lard, and the other ingredients and additives for 2 min in the mixer at 4 °C.
5. Add the starter culture and the probiotic culture at 10^8 CFU/g. Mix for about 2 min (*See* **Notes 1–3**).
6. After a resting phase of 24 h at 0–2 °C, the mixture must be filled using a vacuum filler (*See* **Note 4**).

7. Clip the salami and spray an aqueous mold solution on the surface wrap (*See* **Note 5**).
8. Hang the salami and transfer it to the fermentation chamber (*See* **Note 6**).
9. In the fermentation chamber, the dripping phase occurs at 20 °C for 14–20 h.
10. Keep the salami in the drying room at 20 °C, relative humidity 60 to 80% for 96 to 144 h (*See* **Note 7**).
11. Finally, after drying, transfer the salami to the ripening room at 12–18 °C for 23 days (*See* **Note 8**).
12. The salami can be washed and, thus, packaged in packs without light and oxygen permeability (*See* **Note 9**).

4 Notes

1. The starter culture commonly used in salami is *Staphylococcus xylosus*, which produces lipolytic and proteolytic activity enzymes that are fundamental in the formation and color stability of the final product, and is involved in aroma formation; *Latilactobacillus sakei* with fermenting action, producer of lactic acid and antibacterial metabolites, has also protective action; and *Staphylococcus carnosus*, which adds flavor, has protective and fermenting action.
2. At the end of this stage, the temperature of the mixture rises to about 6 °C.
3. The lactic acid bacteria lower the pH and produce aromatic compounds in the sausage, also masking the bitterness of the curing salts. In addition, they produce reducing conditions, helping to not develop oxidized flavors, and improving color, since they favor the development of meat pigments by stabilizing Fe^{2+}. The also protect the pigments from oxidation by blocking the formation of undesirable compounds in the product.
4. To avoid air residue in the meat paste, it is very important that no air be trapped in the salami.
5. Optionally smoking can be done, but knowing the bacteriostatic effect it can have on some probiotics, this step is not recommended for probiotic Salami.
6. Until pH 4.6–5.4 is reached and for color development.
7. The time of the drying step is given by the weight loss function chosen as the target, which in turn depends on the quality of the lean meat fraction used. If an initial step aimed at losing water from fresh meat is performed in ventilation systems

before grinding, with the objective of losing moisture, the next drying step can be shorter. At the end of the drying stage, the temperature of the drying environment is usually a third lower than it was at the beginning.

8. The length of the ripening chamber will depend on the targeted weight loss. A time of 23 days is sufficient to lower the water activity ($a_w < 0.9$) and achieve the physical-chemical characteristics of *Friolano Salami*. The weight loss at the end of ripening (intended as a complete cycle) is about 38%. This weight loss value can vary according to the lean to fat ratio, diameter, salt concentration, etc. If the previous drying phase has been carried out correctly in terms of weight loss, during the first days of maturation some mold colonies appear on the surface of the wrap. However, this step must be carefully considered since the lower a_w can lead to the loss of probiotic viability.

9. The viability of probiotics over storage depends on the individual strain. Some examples of viability time in storage can be seen in Table 1.

Acknowledgments

The authors are grateful to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES)—Finance Code 001.

References

1. Połka J et al (2017) Bacterial diversity in typical Italian salami at different ripening stages as revealed by high-throughput sequencing of 16S rRNA amplicons. Food Microbiol 46: 342–356, 2015. https://doi.org/10.1016/j.fm.2014.08.023
2. Cenci-Goga BT, Karama M, Sechi P, Iulietto MF, Grispoldi L, Selvaggini R et al (2018) Fate of selected pathogens in spiked «SALAME NOSTRANO» produced without added nitrates following the application of NON-ITTM technology. Meat Sci 139:247–254. https://doi.org/10.1016/j.meatsci.2018.02.002
3. Manassi CF et al (2022) Functional meat products: trends in pro-, pre-, syn-, para- and post-biotic use. Food Res Int 154:111035. https://doi.org/10.1016/j.foodres.2022.111035
4. Thøgersen R, Bertram HC (2021) Reformulation of processed meat to attenuate potential harmful effects in the gastrointestinal tract – a review of current knowledge and evidence of health prospect. Trends Food Sci Technol 108: 111–118. https://doi.org/10.1016/j.tifs.2020.12.015
5. Mafra JF, Cruz AIC, De Santana TS, Ferreira MA, Araújo FM, Evangelista-Barreto NS (2021) Probiotic characterization of a commercial starter culture used in the fermentation of sausages. Food Sci Technol 41(1):240–246. https://doi.org/10.1590/fst.12120
6. Frakolaki G, Giannou V, Kekos D, Tzia C (2021) A review of the microencapsulation techniques for the incorporation of probiotic bacteria in functional foods. Crit Rev Food Sci Nutr 61(9). https://doi.org/10.1080/10408398.2020.1761773
7. Gelinski JMLN, Baratto CM, Casagrande M, Oliveira TP, Megiolaro F, Martini Soares FAS, Souza EMB, Vicente VA, Fonseca GG (2019) Control of pathogens in fresh pork sausage by inclusion of *lactobacillus sakei* BAS0117. Can J Microbiol 65(11):831–841. https://doi.org/10.1139/cjm-2019-0136
8. Ruiz JN, Villanueva NDM, Favaro-Trindade CS, Contreras-Castillo CJ (2014)

Physicochemical, microbiological and sensory assessments of Italian salami sausages with probiotic potential. Sci Agric 71:204–211. https://doi.org/10.1590/S0103-90162014000300005

9. Neffe-Skocińska K, Okoń A, Zielińska D, Szymański P, Sionek B, Kołożyn-Krajewska D (2020) The possibility of using the probiotic starter culture *Lacticaseibacillus rhamnosus* LOCK900 in dry fermented pork loins and sausages produced under industrial conditions. Appl Sci 2020(10):4311. https://doi.org/10.3390/app10124311
10. Wójciak KM, Libera J, Stasiak DM, Kołożyn-Krajewska D (2016) Technological aspect of *Lactobacillus acidophilus* Bauer, *Bifidobacterium animalis* BB-12 and *Lactobacillus rhamnosus* LOCK900 USE in dry-fermented pork neck and sausage. J Food Process Preserv 41(3). https://doi.org/10.1111/jfpp.12965
11. Sameshima T, Magome C, Takeshita K, Arihara K, Itoh M, Kondo Y (1998) Effect of intestinal Lactobacillus starter cultures on the behaviour of Staphylococcus aureus in fermented sausage. Int J Food Microbiol 41(1):1–7. issn:0168-1605. https://doi.org/10.1016/S0168-1605(98)00038-5
12. Rubio R, Jofré A, Martín B, Aymerich T, Garriga M (2014) Characterization of lactic acid bacteria isolated from infant faeces as potential probiotic starter cultures for fermented sausages. Food Microbiol 38:303–311. issn:0740-0020. https://doi.org/10.1016/j.fm.2013.07.015
13. Rubio R, Jofré A, Aymerich T, Guàrdia MD, Garriga M (2014) Nutritionally enhanced fermented sausages as a vehicle for potential probiotic *Lactobacilli delivery.* Meat Sci 96(2, Part A):937–942. issn:0309-1740. https://doi.org/10.1016/j.meatsci.2013.09.008
14. De-Carvalho CCP (2014) Aplicação e viabilidade de micro-organismos potencialmente probióticos em embutidos cárneos fermentados. Universidade Estadual Paulista, São José do Rio Preto, *[s. l.]*, 2014. UNESP
15. Roselino MN (2016) Desenvolvimento de um embutido cárneo fermentado, com teores reduzidos de gordura e sais de cura, através da utilização de culturas probióticas. Universidade Estadual Paulista, Araraquara, *[s. l.]*, 2016. Disponível em: http://hdl.handle.net/11449/138151
16. Rubio R, Martín B, Aymerich T, Garriga M (2014) The potential probiotic *Lactobacillus rhamnosus* CTC1679 survives the passage through the gastrointestinal tract and its use as starter culture results in safe nutritionally enhanced fermented sausages. Int J Food Microbiol 186:55–60. issn:0168-1605. https://doi.org/10.1016/j.ijfoodmicro.2014.06.013
17. Mafra JF, Cruz AIC, Santana TS, Ferreira MA, Araújo FM, Evangelista-Barreto NS (2020) Probiotic characterization of a commercial starter culture used in the fermentation of sausages. https://doi.org/10.1590/fst.12120
18. Trząskowska M, Kołożyn-Krajewska D, Wójciak K, Dolatowski Z (2014) Microbiological quality of raw-fermented sausages with Lactobacillus casei LOCK 0900 probiotic strain. Food Control 35(1):184–191. issn:0956-7135. https://doi.org/10.1016/j.foodcont.2013.07.002
19. Holko I, Hrabě J, Šalaková A, Rada V (2013) The substitution of a traditional starter culture in mutton fermented sausages by Lactobacillus acidophilus and Bifidobacterium animalis. Meat Sci 94(3):275–279, issn:0309-1740. https://doi.org/10.1016/j.meatsci.2013.03.005
20. Klingberg TD, Axelsson L, Naterstad K, Elsser D, Budde BB (2005) Identification of potential probiotic starter cultures for Scandinavian-type fermented sausages. Int J Food Microbiol 105(3):419–431. https://doi.org/10.1016/j.ijfoodmicro.2005.03.020
21. Erkkilä S, Suihko ML, Eerola S, Petäjä E, Mattila-Sandholm T. (2001) Dry sausage fermented by lactobacillus rhamnosus strains. Int J Food Microbiol 2001;64(1–2):205–210. https://doi.org/10.1016/s0168-1605(00)00457-8. PMID: 11252505
22. Agüero NDL, Frizzo LS, Ouwehand AC, Aleu G, Rosmini MR (2020) Technological characterisation of probiotic lactic acid bacteria as starter cultures for dry fermented sausages. Foods 9(5):596. https://doi.org/10.3390/foods9050596
23. Ruiz-Moyano S, Martín A, Benito MJ, Aranda E, Casquete R, de Guia Córdoba M (2011) Implantation ability of the potential probiotic strain, Lactobacillus reuteri Pl519, in "salchichón," a traditional Iberian dry fermented sausage. J Food Sci 76(5):M268–M275. https://doi.org/10.1111/j.1750-3841.2011.02189.x
24. Essid I, Hassouna M (2013) Effect of inoculation of selected Staphylococcus xylosus and Lactobacillus plantarum strains on biochemical, microbiological and textural characteristics of a Tunisian dry fermented sausage. Food Control 32(2):707–714. https://doi.org/10.1016/j.foodcont.2013.02.003
25. Han Q, Kong B, Chen Q, Sun F, Zhang H (2017) In vitro comparison of probiotic properties of lactic acid bacteria isolated from

Harbin dry sausages and selected probiotics. J Funct Foods 32:391–400. https://doi.org/10.1016/j.jff.2017.03.020

26. Özer CO, Kılıç B, Kılıç GB (2016) In-vitro microbial production of conjugated linoleic acid by probiotic L. plantarum strains: utilization as a functional starter culture in sucuk fermentation. Meat Sci 114:24–31. https://doi.org/10.1016/j.meatsci.2015.12.005

27. Blaiotta G, Murru N, Di Cerbo A, Romano R, Aponte M (2018) Production of probiotic bovine salami using Lactobacillus plantarum 299v as adjunct. J Sci Food Agric 98(6):2285–2294. https://doi.org/10.1002/jsfa.8717

28. Cenci-Goga BT, Karama M, Sechi P, Iulietto MF, Grispoldi L, Selvaggini R et al (2018) Fate of selected pathogens in spiked «SALAME NOSTRANO» produced without added nitrates following the application of NONIT™ technology. Meat Sci 139:247–254. https://doi.org/10.1016/j.meatsci.2018.02.002

29. de Macedo REF, Pflanzer SB Jr, Terra NN, De Freitas RJS (2008) Production of fermented sausage using probiotic lactobacillus strains: quality characteristics. Ciênc Tecnol Aliment Campinas 28(3):509–519

Chapter 12

Probiotic in Bakery

Ana Paula Zapelini de Melo, Thais de Oliveira, Pedro Luiz Manique Barreto, and Silvani Verruck

Abstract

The incorporation of probiotic microorganisms (such as the genera *Bifidobacterium*, *Lactobacillus*, *Lactiplantibacillus*, *Lacticaseibacillus*, and *Bacillus*) into bakery products is an emerging approach to enlarge the availability of non-dairy probiotic food on the market. However, the viability and stability of the microorganisms may be compromised due to the high processing temperatures employed in the development of these products. This chapter proposes the design of two independent protocols for the delivery of probiotics through bakery products: (I) a probiotic bread by adding microorganisms directly to the dough and (II) an edible probiotic film based on sodium caseinate and chia mucilage for application on bread surface. Furthermore, for both protocols, the function of each reagent/ingredient and the chemical reactions involved are described in details, indicating the sources of possible issues and the best alternatives to overcome them.

Key words Bread ingredients, Functional foods, Probiotic bread, Probiotic edible film, Probiotic cultures, Non-dairy probiotic

1 Introduction

Probiotics are defined as live microorganisms that provide benefits to the human health when properly administered [1]. For decades, dairy products such as yogurts and cheeses were the main matrices used for the delivery of probiotics. However, the increase in lactose intolerant and allergic individuals, combined with the evidence of beneficial effects of probiotic microorganisms on human health, has propelled the development of new non-dairy probiotics products [2].

An emerging approach to expand the availability of non-dairy probiotics food is the incorporation of these microorganisms in bakery products [3–8]. The development of probiotic bakery products is a challenge, due to the high processing temperatures that they are subjected to (between 160 and 250 °C), which may compromise the viability and stability of the microorganisms used

Adriano Gomes da Cruz et al. (eds.), *Probiotic Foods and Beverages: Technologies and Protocols*, Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-3187-4_12,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2023

[9]. To ensure the beneficial effects on human health, especially related to gastrointestinal health, a minimum concentration of live probiotic cells must reach the intestine. The suggested minimum concentration ranges from 10^8 to 10^9 CFU/dose [1]. However, for some products, lower concentrations are sufficient to claim specific health effects, while for others, a substantially higher concentration is required, depending on the strain [1, 2].

The main microorganisms with stated probiotic properties used in bakery products include the genera *Bifidobacterium*, *Lactobacillus*, *Lactiplantibacillus*, and *Lacticaseibacillus*. Nonetheless, to overcome the limitations associated with the processing and storage of the products, as well as to the gastrointestinal conditions, encapsulation techniques may be used as an alternative to protect cell viability under unfavorable environmental conditions (*see* Chapter 14 for detailed information regarding the encapsulation of probiotic microorganisms) [10]. On the other hand, spore-forming probiotic strains, such as those of the genus *Bacillus*, have recently overcome the technological challenges related to stressful conditions of bakery products processing (such as high temperature survival) without the need for encapsulation [5, 8].

The development of probiotic bakery products is still in the early stages. Most of the studies published in the literature are directed to the development of probiotic breads. In fact, bread is an interesting matrix, especially due to its large consumption worldwide [11]. Protocols for the delivery of probiotics through breads vary considerably in the literature; however, promising strategies include, but are not limited to, the addition of microorganisms in dough and as edible coating films [12, 13]. Therefore, this chapter is directed to the design of a probiotic bread protocol through the addition of probiotic microorganisms directly to the dough and, in parallel, an edible film protocol of probiotic coating based on sodium caseinate and chia mucilage for application on bread surface.

2 Materials

2.1 Probiotic Microorganisms

1. The main microorganisms with claimed probiotic properties used in bakery products are presented in Table 1 (*see* **Note 1**).

2.2 Edible Film

1. Distilled water.
2. Sodium caseinate.
3. Glycerol.
4. Chia mucilage.
5. Probiotic culture.

Table 1
Main microorganisms with claimed probiotic properties used in bakery products

Genus	Species	Strains
Bacillus	*Coagulans subtilis*	GBI-306086 [5, 8] MTCC 5856 [5]
Bifidobacterium	*Bifidum lactis*	BB-12 [14] and NCDC 236 [15] NH019 [6]
Lactobacillus	*Acidophilus*	LA-5 [5, 14, 16], PTCC 1643 [17], and NCDC 11 [15]
Lactiplantibacillus	*Plantarum*	P8 [3]
Lacticaseibacillus	*Rhamnosus*	GG [7] and NCDC 17 [15]

2.3 Bread

1. Wheat flour.
2. Water.
3. Sucrose.
4. Sodium chloride.
5. Active dry yeast (*Saccharomyces cerevisiae*).
6. Fat.
7. Probiotic culture.

2.4 Equipment

1. Magnetic stirrer.
2. Sonicator (such as VC 505, Sonics & Materials Inc., USA).
3. Centrifuge.
4. Cheesecloth.
5. Vacuum degasser.
6. Pastry brush.
7. Kneading-trough (such as A30 Progress, Brazil).
8. Proofing chamber (such as MSV 750, Heidenreich, Germany).
9. Convection oven (such as Vipinho-0448, Perfecta, Brazil).

3 Methods

The designs of two independent protocols for the delivery of probiotics through bakery products are presented in Fig. 1: (I) a probiotic bread by adding microorganisms directly to the dough and (II) an edible probiotic film based on sodium caseinate and chia mucilage for application on bread surface.

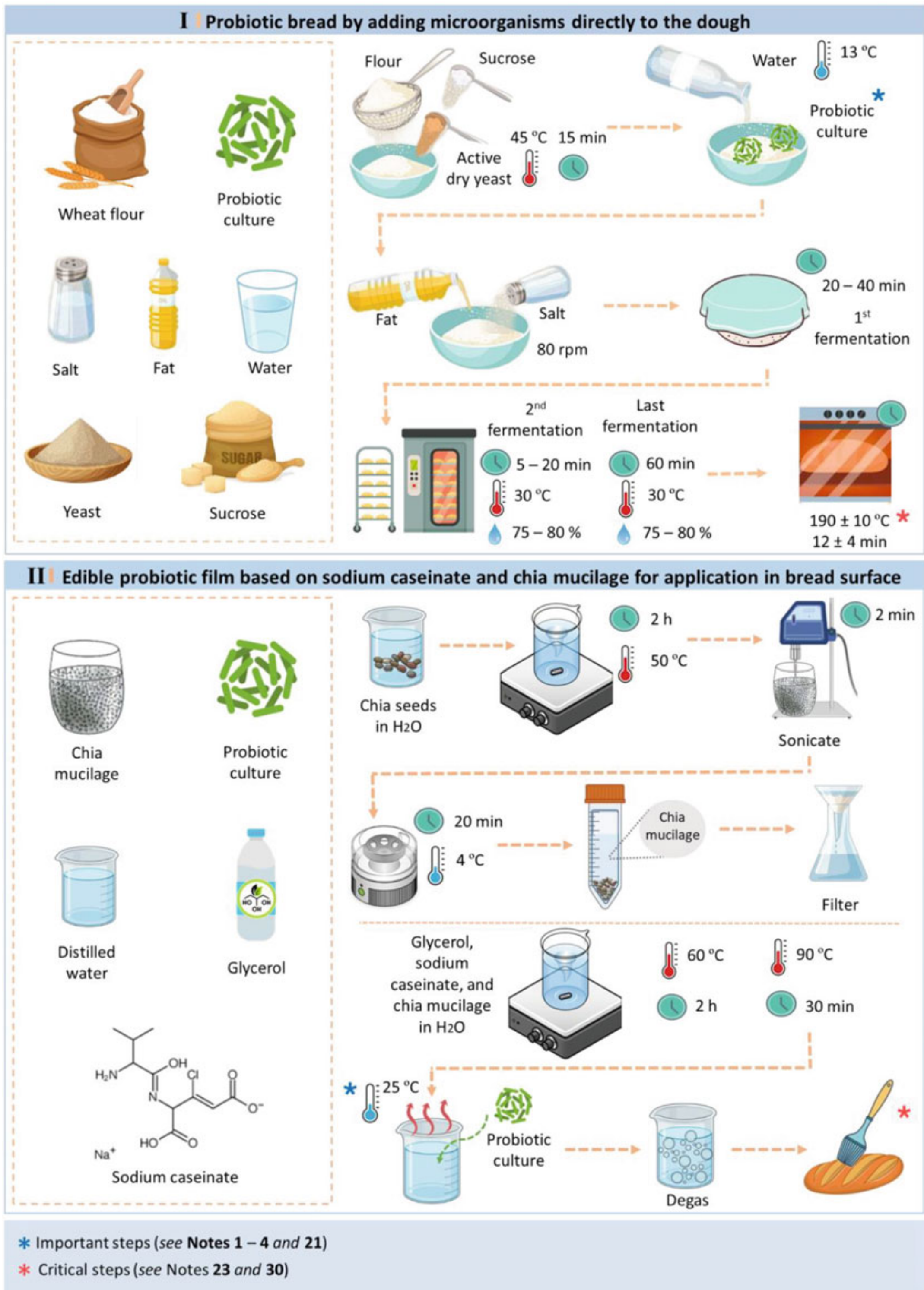

Fig. 1 Steps for the delivery of probiotics through bakery products: (I) a probiotic bread by adding microorganisms directly to the dough and (II) an edible probiotic film based on sodium caseinate and chia mucilage for application on bread surface

3.1 Probiotic Cultures

1. Use *direct vat set* (DVS) or *direct vat inoculation* (DVI) cultures that allow the addition of probiotics directly to the food matrix, without the need for activation or propagation of the microbial culture in specific fermentation bulk (*see* in Chapter 13 the main exporter suppliers of DVS probiotic strains) [18] (*see* **Notes 2–4**).

3.2 Probiotic Bread

1. Mix dry ingredients (except sodium chloride) at 40 rpm for 1 min. Ingredients are calculated based on the amount of flour (100%):
   - Wheat flour (100%) (*see* **Note 5**).
   - Sucrose (4%) (*see* **Notes 6** and 7).
   - Active dry yeast (*Saccharomyces cerevisiae*) (2%) in warm water (45–55 °C, until it reaches five times its own weight, for 15–20 min) (*see* **Notes 8** and **9**).
2. Add water (60%) between 4 and 13 °C and mix at 40 rpm for 1 min and then at 80 rpm for 7 min (*see* **Notes 10–11**).
3. Add 10^9–10^{11} CFU/g of lyophilized probiotic culture [19] (*see* **Notes 1–3**).
4. Add sodium chloride (2%) (*see* **Note 12**).
5. Add the fat source (3%) under 40 rpm speed (*see* **Note 13**).
6. When homogeneity is reached, increase the speed of the kneading-trough to 80 rpm to start the kneading process (*see* **Notes 14–16**).
7. Let the dough rest for 20–40 min (first fermentation) on a surface lightly oiled and covered with plastic, to prevent the dough from drying out, and then carry out the dough division process (*see* **Note 17**).
8. Perform the dough rounding and let the breads rest in a proofing chamber (second fermentation) at 26–30 °C, relative humidity 75–80%, for a minimum of 5 and a maximum of 20 min (*see* **Notes 18** and **19**).
9. Mould the dough pieces and return them to the chamber for the last stage of fermentation, 26–30 °C, relative humidity 75–80%, for 60 min (*see* **Notes 20** and **21**).
10. Bake at 190 ± 10 °C for 12 ± 4 min in a convection oven (*see* **Notes 22** and **23**).

3.3 Probiotic Edible Film

3.3.1 Chia (*Salvia hispanica L.*) Mucilage Extraction

1. Hydrate the chia seeds in 1:30 distilled water (seed:water, w/w) for 2 h at 50 °C under magnetic stirring (600 rpm) (*see* **Note 24**).
2. Sonicate the solution for 2 min at 500 W, 20 kHz and 30% amplitude (*see* **Note 25**).

3. Centrifuge at 3488-*g* at 4 °C for 20 min (*see* **Note 26**).
4. Collect the chia mucilage (middle layer).
5. Filter mucilage through cheesecloth.

3.3.2 Probiotic Edible Film Based on Sodium caseinate and Chia Mucilage

1. Heat distilled water at 50 °C.
2. Under magnetic stirring (600 rpm), add sodium caseinate (5%, w/w) until complete dissolution (*see* **Notes 27** and **28**).
3. Add glycerol (30%, w/w) and chia mucilage (1%, w/w), and keep it under magnetic stirring (600 rpm) until dissolution (*see* **Note 29**).
4. Heat the solution at 60 °C for 2 h and then at 90 °C for 30 min, both under magnetic stirring (600 rpm).
5. Cool the polymeric solution to room temperature and add 10^9–10^{11} CFU/g of lyophilized probiotic culture under magnetic stirring (600 rpm) [19] (*see* **Notes 1–3** and **30**).
6. Degas the solution under vacuum for 20 min (*see* **Note 31**).
7. After baking the bread, wait for them to cool down and apply with brushing using a pastry brush a thin layer of the probiotic polymeric solution over their crust (*see* **Note 32**).

3.3.3 Other Methods for Delivering Probiotics Through Bread

The main conditions for the delivery of probiotics through breads available in the literature are summarized in Table 2.

Table 2
Main conditions for delivery of probiotics through breads

Probiotic strain	Inoculation dose	Inoculation conditions	Baking conditions	Storage conditions	Probiotic viability after bake (CFU/g)	References
Bacillus coagulans GBI-30, 6086	10^7 spores/g	DVS	200 °C18 min	25 °C 10 days	10^4 (after 10 days)	[8]
B. coagulans GBI-306086 *B. coagulans* MTCC 5856 *B. subtilis* PXN 21	10^7–10^8 CFU/g	MYP 37 °C/24 h and 20 °C/24 h BC 40 °C/48 h GYEA 37 °C/72 h	180 °C 20 min	25 °C 7 days	10^3–10^6 (after 7 days)	[5]

(continued)

Table 2 (continued)

Probiotic strain	Inoculation dose	Inoculation conditions	Baking conditions	Storage conditions	Probiotic viability after bake (CFU/g)	References
Lactiplantibacillus plantarum P8	10^9 CFU/g	MRS 37 °C/12 h and 37 °C/24 h	175 °C 8 min	25 °C 5 days	10^6 (after 5 days)	[3]
Lactobacillus acidophilus LA-5 (encapsulated with alginate and fish gelatin)	10^9 CFU/g	MRS 37 °C/12 h and 37 °C /24 h.	175 °C 6 min	25 °C 7 days	10^6 (after 7 days)	[16]
Lactiplantibacillus plantarum P8 (encapsulated with reconstituted skim milk)	10^9 CFU/g	MRS 37 ° C/12 h and 37 °C/24 h	100 °C 15 min	–	10^8	[4]
Lactobacillus acidophilus PTCC 1643 (encapsulated with alginate and chitosan)	10^9 CFU/ mL	MRS 37 °C/24 h and 37 °C/48 h	180 °C35 min	–	–	[17]
Bifidobacterium animalis spp. *lactis* NH019 (encapsulated with stearic acid, hydroxypropyl cellulose, and sodium alginate)	10^{11} CFU/g	DVS	180 °C 40 min	–	10^5	[6]
Lacticaseibacillus rhamnosus GG (encapsulated with sodium alginate, hi-maize resistant starch, and chitosan)	–	MRS 37 °C/48 h	220 °C 20 min	–	–	[7]

–: Not described, *DVS* Direct vat set, *MRS De Man, Rogosa & Sharpe Agar*, *MYP Mannitol Egg Yolk Polymyxin Agar*, *BC Glucose Yeast Extract Agar*, *GYEA* Glucose Yeast Extract Agar

4 Notes

1. The microorganisms of the genera *Bifidobacterium*, *Lactobacillus*, *Lactiplantibacillus*, and *Lacticaseibacillus* are gram-positive microorganisms with thick cell walls, being able, therefore, to withstand most of the forces generated in bread production processes. However, high homogenization speeds can result in cell disruption and reduced cell viability. Furthermore, these genera are not able to survive extreme temperature conditions, therefore, use encapsulation techniques for proper delivery through breads (*see* Chapter 14). *Bacillus* genus microorganisms (such as *B. coagulans* GBI-30, 6086 spores) can withstand typical bread processing conditions; thus, they do not require encapsulation [9, 18, 19].
2. The addition of 10^9–10^{11} CFU/g of the probiotic culture in food is recommended to reach the suggested minimum dose (10^8–10^9 CFU/dose of food) to promote human health benefits. This concentration usually represents the addition of 0.1% of probiotic in the final product [19].
3. When the direct addition of probiotics to the food matrix results in losses in cell viability, a rehydration step must be carried out and, subsequently, the rehydrated cell suspension must be added to the food matrix. Rehydration is usually performed in skim milk; however, water, saline solution, or liquid culture medium can also be used depending on the strain. The suggested rehydration temperature for thermophiles is between 30 and 37 °C, while for mesophiles it is 22–30 °C [18].
4. Store probiotics following the manufacturer's instructions to preserve cell viability by protecting them from light and moisture content at a constant temperature [18].
5. The strength of the flour has effects on the duration of the fermentation time and is related to its protein content, therefore, to achieve optimal dough development (larger volume and soft crumb), use flours with $13 \pm 1\%$ of protein content for adequate gas retention [20].
6. Sucrose, besides conferring flavor and browning to the crust of breads (Maillard reaction), controls water activity and prolongs the shelf life of the products [20].
7. Sucrose concentrations below 10% generally do not inhibit probiotic microorganisms [18].
8. The fermentation is an anaerobic process produced by the action of the yeast on the sugar present in the bread dough. This step is responsible for the production of carbon dioxide and small quantities of ethyl alcohol, along with the

physicochemical transformations that alter the viscoelastic properties of the dough. The starch present in the flour is converted into sugars though enzymatic reactions, and the resulting sugars from this reaction feed the yeast and outcome in the formation of carbon dioxide [20, 21].

9. Yeasts are inactivated at 55 °C or higher temperatures, therefore do not exceed this temperature [20].
10. Water must be between 4 and 13 °C to prevent the early start of the fermentation process, intermediate hardness between 50 and 100 mg L^{-1} of calcium carbonate and pH approximately 7.0 [21, 22].
11. The speed of 40 rpm is used for the incorporation of the ingredients, while the speed of 80 rpm is indicated for the gluten development and the beginning of the kneading process [16].
12. Sodium chloride, in addition to its contributions to the flavor of the final product and controlling water activity, affects the hydration rate of gluten proteins and inhibits yeast fermentation due to cell dehydration (osmotic pressure). Therefore, incorporate sodium chloride in the final steps of the mixing process. The absence of sodium chloride results in an excess of softening in the dough, rapid fermentation, and reduction of the volume of the product, while its excess leads to a reduction in the fermentative action, causing the gluten to harden excessively [20, 23].
13. Lipids delay the retrogradation of starch gels and provide softness to baked goods, due to interactions between the lipid micelles with the hydrophilic segments of the starch [24]. For a homogeneous dispersion of the fat in the dough, use saturated fats, with a chain length of C_{16}–C_{18} (tripalmitin and tristearin) and with a high melting point (55–60 °C) [20].
14. During the mixing process, under application of tension and shear forces, the gluten proteins are hydrated, providing the necessary energy for the development of the gluten structure. Gluten proteins leave their ball-like shape and acquire a linear shape, facilitating hydrophobic interactions and sulfhydryl-disulfide interchange reactions. This results in the formation of threadlike polymers, which in turn, through hydrogen bonds, disulfide cross-links, and hydrophobic associations, form a film capable of retaining the gas in the dough [22, 24].
15. For proper gluten development, the dough must be between 27 and 29 °C at the end of the mixing stage. Excessive heating of the dough can compromise fermentation, which will be stimulated in advance, affecting the bread growth and the gluten viscoelastic properties [20, 22, 23].

16. Excessive kneading leads to gluten breakdown, and the lack of it leads to absence of elasticity in the dough [20, 23].
17. The doughs that do not undergo the resting process have considerably smaller volumes of gas in their composition, influencing the subsequent operations of modeling and dough weight control. Care must be taken when dividing, as any compression of the dough during this process will cause a reduction in the regularity of the weight of the product due to its degassing, that is, leads to variations in the gaseous volumes retained in the dough, thus causing damage to its structure [20].
18. Post-rounding fermentation (second fermentation) assists on the recovery of dough extensibility, lost during the division and rounding processes. Optimum temperature at this stage varies between 26 and 30 °C, with a relative humidity of 75–80%. Temperatures higher than these reduce the gas holding capacity, while lower temperatures delay the fermentation. Proofing chambers with low relative humidity cause the dough to dry out and the consequent formation of a crust, while higher humidity increases the stickiness of the dough, making it difficult to manipulate [20, 21].
19. Insufficient bulk time provides tough, rubbery, and not easily moldable gluten, and consequently breads of small volume, firm crumb, and dense cell structure. On the other hand, long bulk times result in the release of gas present in the dough, loss of deformation resistance, and increase in extensibility, and consequently irregular breads with large holes [20, 21].
20. The final stage of fermentation occurs under the same conditions of the second fermentation, for a longer period of time to allow the dough to regain an adequate size, due to the loss of gas resulting from the moulding step. The fermentation process is finished inside the oven, when the temperature reaches 55 °C [21, 22].
21. The fermentation step does not negatively affect the viability of probiotic microorganisms [8]. The association between probiotic microorganisms and yeasts in the dough during fermentation is steady due to the absence of nutrients competition between these microbial populations. The presence of fermentable carbohydrates throughout the fermentation process allows a stable noncompetitive association, and consequently, an increase in the growth rates of probiotic microorganisms, due to the excretion of essential amino acids by the yeasts. However, for this to occur, the growth rates of microbial populations must be similar and there must be no exhaustion of fermentable carbohydrates during the process [25, 26].

22. Upon reaching approximately 60 °C, the denaturation process of the flour proteins and changes in the structure of the starch begins, increasing the viscosity of the dough. The rise in temperature promotes an increase in the number of disulfide bonds and hydrophobic interactions between nonpolar amino acid residues in flour proteins, providing stability to the bread structure [24].
23. The bread baking step is the most critical, as high temperatures can result in reduced viability and stability of probiotic microorganisms. In addition to encapsulation techniques (*see* **Note 1** and Chapter 14), paraprobiotics are an emerging strategy (*see* Chapter 15). Paraprobiotics are defined as cells (or cell fractions) of nonviable microorganisms that can provide benefits to human health when administered in adequate amounts [27]. However, there are no studies available in the literature using bakery products for the delivery of paraprobiotics, making it a possible field to be explored.
24. For complete water absorption by chia seeds, hydrate them until constant weight (around 2 h).
25. Sonication propagates acoustic waves through chia seeds, generating cavitation bubbles, that through physical and chemical effects, break the matrix and promote the transfer of the mucilaginous gel firmly adhered in the seeds to the solvent (water) [28].
26. In this step, three layers are formed: (I) the lower layer, which contains the chia seeds; (II) the middle layer to the chia mucilage; and (III) the layer above water.
27. Sodium caseinate must be properly dissolved in the solution to avoid lumps or particles that could affect the structural integrity of the film [29].
28. The aleatory coil structure and interactions with molecules coming from hydrogen and hydrophobic bonds, and electrostatic force give sodium caseinate film-forming characteristics. In addition, the high proportion of polar groups in sodium caseinate provides adequate barrier capacity for oxygen, carbon dioxide, and aromatic compounds present in the environment [29].
29. The combination of the plasticizer glycerol and chia mucilage provides the films with thermal resistance, a barrier to light and oxygen, and flexibility due to the reduction of intermolecular attractions between nearby polymer chains. Furthermore, the hydrophilic nature of chia mucilage, due to heteropolysaccharides with hydrophilic groups, protects microbial cells against environmental stress, and therefore maintains the viability of the probiotics [13, 30].

30. Cooling to room temperature ensures sufficient time for microorganism cells to adapt to the water activity transition, maintaining the viability of the probiotics [13].
31. Air must be removed to prevent the formation of bubbles that could compromise the structural integrity of the film [13].
32. The probiotic coating film does not have the function of acting as packaging. Therefore, pack the bread coated with probiotic edible films in polyethylene bags at room temperature (approximately 25 °C) for 7 days.

Acknowledgments

The authors are grateful to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES)—Finance Code 001.

References

1. WGO (2017) Probiotics and prebiotics. World Gastroenterology Organisation, Milwaukee
2. Pimentel TC, da Costa WKA, Barão CE et al (2021) Vegan probiotic products: a modern tendency or the newest challenge in functional foods. Food Res Int 140:110033. https://doi.org/10.1016/j.foodres.2020.110033
3. Zhang L, Taal MA, Boom RM et al (2018) Effect of baking conditions and storage on the viability of *Lactobacillus plantarum* supplemented to bread. LWT Food Sci Technol 87:318–325. https://doi.org/10.1016/j.lwt.2017.09.005
4. Zhang L, Chen XD, Boom RM, Schutyser MAI (2018) Survival of encapsulated *Lactobacillus plantarum* during isothermal heating and bread baking. LWT Food Sci Technol 93:396–404. https://doi.org/10.1016/j.lwt.2018.03.067
5. Soares MB, Martinez RCR, Pereira EPR et al (2019) The resistance of *Bacillus*, *Bifidobacterium*, and *Lactobacillus* strains with claimed probiotic properties in different food matrices exposed to simulated gastrointestinal tract conditions. Food Res Int 125:108542. https://doi.org/10.1016/j.foodres.2019.108542
6. Penhasi A, Reuveni A, Baluashvili I (2021) Microencapsulation may preserve the viability of probiotic bacteria during a baking process and digestion: a case study with *Bifidobacterium animalis* subsp. *lactis* in bread. Curr Microbiol 78:576–589. https://doi.org/10.1007/s00284-020-02292-w
7. Ezekiel OO, Okehie ID, Adedeji OE (2020) Viability of *Lactobacillus rhamnosus* GG in simulated gastrointestinal conditions and after baking white pan bread at different temperature and time regimes. Curr Microbiol 77:3869–3877. https://doi.org/10.1007/s00284-020-02203-z
8. Almada-Érix CN, Almada CN, Souza Pedrosa GT et al (2022) Bread as probiotic carriers: resistance of *Bacillus coagulans* GBI-30 6086 spores through processing steps. Food Res Int 155. https://doi.org/10.1016/j.foodres.2022.111040
9. Arepally D, Reddy RS, Goswami TK, Coorey R (2022) A review on probiotic microencapsulation and recent advances of their application in bakery products. Food Bioprocess Technol. https://doi.org/10.1007/s11947-022-02796-2
10. Manassi CF, de Souza SS, de Hassemer GS et al (2022) Functional meat products: trends in pro-, pre-, syn-, para- and post-biotic use. Food Res Int 154:111035. https://doi.org/10.1016/j.foodres.2022.111035
11. Cauvain SP, Clark RH (2019) Baking technology and nutrition. Wiley, Hoboken
12. De Prisco A, Mauriello G (2016) Probiotication of foods: a focus on microencapsulation tool. Trends Food Sci Technol 48:27–39. https://doi.org/10.1016/j.tifs.2015.11.009
13. Semwal A, Ambatipudi K, Navani NK (2022) Development and characterization of sodium caseinate based probiotic edible film with chia mucilage as a protectant for the safe delivery of probiotics in functional bakery. Food Hydrocoll Heal 2:100065. https://doi.org/10.1016/j.fhfh.2022.100065

14. Arslan-Tontul S, Erbas M, Gorgulu A (2019) The use of probiotic-loaded single- and double-layered microcapsules in cake production. Probiotics Antimicrob Proteins 11:840–849. https://doi.org/10.1007/s12602-018-9467-y
15. Muzzafar A, Sharma V (2018) Microencapsulation of probiotics for incorporation in cream biscuits. J Food Meas Charact 12:2193–2201. https://doi.org/10.1007/s11694-018-9835-z
16. Hadidi M, Majidiyan N, Jelyani AZ et al (2021) Alginate/fish gelatin-encapsulated *Lactobacillus acidophilus*: a study on viability and technological quality of bread during baking and storage. Foods 10:2215. https://doi.org/10.3390/foods10092215
17. Mirzamani SS, Bassiri AR, Tavakolipour H et al (2021) Survival of fluidized bed encapsulated *Lactobacillus acidophilus* under simulated gastro-intestinal conditions and heat treatment during bread baking. J Food Meas Charact 15:5477–5484. https://doi.org/10.1007/s11694-021-01108-0
18. Charalampopoulos D, Rastall RA (2009) Prebiotics and probiotics science and technology. Springer, Cham
19. Lee YK, Salminen S (2009) Handbook of probiotics and prebiotics, 2nd edn. Wiley, Hoboken
20. Cauvain S (2015) Technology of breadmaking, 3rd edn. Springer, Cham
21. Aquarone E, Borzani W, Schmidell W, de Lima UA (2001) Biotecnolgia industrial, 2nd edn. Blucher, São Paulo
22. ABNT, SEBRAE (2015) Guia de implementação panificação – pão tipo francês – Diretrizes para avaliação da qualidade e classificação – ABNT NBR 16170. Associação Brasileira de Normas Técnicas eServiço Brasileiro de Apoio às Micro e Pequenas Empresas, Rio de Janeiro
23. Vianna FSV, Redoschi G, Lage M et al (2020) Manual prático de panificação. Senac, São Paulo
24. Damodaran S, Parkin KL (2017) Fennema's food chemistry, 5th edn. CRC Press, Boca Raton
25. Jin J, Nguyen TTH, Humayun S et al (2021) Characteristics of sourdough bread fermented with *Pediococcus pentosaceus* and *Saccharomyces cerevisiae* and its bio-preservative effect against Aspergillus flavus. Food Chem 345:128787. https://doi.org/10.1016/j.foodchem.2020.128787
26. Venturi M, Guerrini S, Vincenzini M (2012) Stable and non-competitive association of Saccharomyces cerevisiae, *Candida milleri* and *Lactobacillus sanfranciscensis* during manufacture of two traditional sourdough baked goods. Food Microbiol 31:107–115. https://doi.org/10.1016/j.fm.2012.02.011
27. Taverniti V, Guglielmetti S (2011) The immunomodulatory properties of probiotic microorganisms beyond their viability (ghost probiotics: proposal of paraprobiotic concept). Genes Nutr 6:261–274. https://doi.org/10.1007/s12263-011-0218-x
28. Silva LA, Sinnecker P, Cavalari AA et al (2022) Extraction of chia seed mucilage: effect of ultrasound application. Food Chem Adv 1:100024. https://doi.org/10.1016/j.focha.2022.100024
29. Lin HC, Wang BJ, Weng YM (2020) Development and characterization of sodium caseinate edible films cross-linked with genipin. LWT Food Sci Technol 118:108813. https://doi.org/10.1016/j.lwt.2019.108813
30. Dick M, Costa TMH, Gomaa A et al (2015) Edible film production from chia seed mucilage: effect of glycerol concentration on its physicochemical and mechanical properties. Carbohydr Polym 130:198–205. https://doi.org/10.1016/j.carbpol.2015.05.040

Chapter 13

Probiotic and Synbiotic Chocolate

Milena Dutra Pierezan, Callebe Camelo-Silva, Alan Ambrosi, Marco Di Luccio, and Silvani Verruck

Abstract

Chocolate is a mixture of cocoa by-products, sucrose, and milk solids dispersed in the fat phase (mainly cocoa butter). It consists of an excellent probiotic matrix due to its low water activity and the considerable presence of fat and antioxidant compounds. The *Lactobacillus*, *Lacticaseibacillus*, *Limosilactobacillus*, *Bifidobacterium*, and *Streptococcus* comprehend the most applied genus in probiotic chocolate. Its processing involves the same steps as the traditional chocolate (blending, refining, conching, tempering, molding, cooling, and storage), with the additional step of probiotic inoculation. In most cases, a single Direct Vac Set probiotic inoculum, which consists of a freeze-dried cell concentrate, can be added and homogenized directly into the melted chocolate. Inoculation has to take place after the conching process to ensure cell viability. Currently, there is not a defined general amount of bacteria to be introduced into the food to ensure the probiotic health benefits. It must be proven for each strain and inoculum tested. But in general, cell concentrations between 10^6 and 10^{13} CFU/g are being used to ensure the probiotic claim. Furthermore, there is a current controversy over functional chocolate such as the probiotic options due to their high sugar content. This has motivated researchers and food industries to produce sugar-free and reduced-sugar options. Considering that some sugar substitute ingredients can act as prebiotics, promoting the growth of probiotic microorganisms, the combination of prebiotic and probiotic functionality originated the synbiotic chocolate. Its production also involves the probiotic inoculation step besides the traditional stages of chocolate processing, but instead of sugar, the prebiotic and other sugar-replacer ingredients are added, usually into the blending step with the other main ingredients. Therefore, considering the growing literature involving this theme, the objective of this protocol is to provide up-to-date and detailed information for the production of different types of probiotic and synbiotic chocolate.

Key words Cocoa, Functional chocolate, Low-sugar chocolate, Low-fat chocolate, Probiotic microencapsulation

1 Introduction

Chocolate can be defined as a cocoa by-product (cocoa mass, cocoa powder and/or cocoa liquor), sucrose and milk solids dispersed in the fat phase, which is mainly composed of cocoa butter [1]. Emulsifying agents such as soy lecithin and/or polyglycerol polyricinoleate (PGPR) are also usually added, as well as ethyl vanillin, a

Adriano Gomes da Cruz et al. (eds.), *Probiotic Foods and Beverages: Technologies and Protocols*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-3187-4_13,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2023

flavoring agent [2, 3]. The presence of some ingredients can differ among the different types of chocolate, and other specifications can be defined for the production of chocolate depending on the regulations of each country, which can change the possibilities of product formulation. Furthermore, among innovative functional chocolate products, the probiotic option has been extensively studied [1].

Probiotics are live microorganisms that benefit the host's health, mainly by improving intestinal problems. However, they also can provide other health benefits, such as lowering cholesterol and blood pressure levels and improving mineral absorption and the immune system [4]. In this context, chocolate has been shown to be an effective matrix for the active and viable delivery of probiotics to the gut [5]. The low water activity of chocolate keeps the probiotics in a low metabolic state, increasing their viability in the chocolate matrix during storage. In addition, some cocoa high-fat content by-products can decrease oxygen availability to the probiotic cell, preventing oxidation and protecting cell viability from thermal inactivation during processing [6]. Although phenols may act as antimicrobials, they did not decrease the survival of probiotic bacteria into chocolate [4]. Indeed, more cells tend to remain viable in dark chocolate, which contains more cocoa antioxidant compounds, such as flavonoids, when compared to milk and white chocolate types [7].

In general, the processing of probiotic chocolate involves the same process steps as traditional chocolate (blending, refining, conching, tempering, molding, cooling, and storage), with the additional step of probiotic inoculation. Once the conching step can reach temperatures of up to 70 °C in some cases, probiotic is usually added after this, in order to ensure cell viability [8]. In general, Direct Vac Set (DVS) inoculums, which consist of a concentrate of freeze-dried cells, are used. This probiotic powder can be added directly and homogenized in the melted product [9]. Among all the probiotic genus, the *Lactobacillus*, *Lacticaseibacillus*, *Limosilactobacillus*, *Bifidobacterium*, and *Streptococcus* are the most applied in studies involving chocolate production [7]. In addition, there is not a defined general amount of bacteria to be introduced into the food to ensure the probiotic health benefits. It must be proven for each strain and inoculum tested. But in general, cell concentrations between 10^6 and 10^{13} CFU/g are being used to ensure the probiotic claim [2, 6].

Besides the health benefits of probiotics, there is a controversy over the use of this functionality into chocolate due to its high sugar and calorie content [10]. A high sugar intake is strongly associated with negative implications such as obesity, diabetes, and oral health. Thus, combining sweeteners like sucralose, stevioside, thaumatin, and sugar alcohols with a bulking agent like inulin, maltodextrin, and polydextrose has been widely used to partially or

totally replace sucrose in probiotic chocolate. Interestingly, some of these sugar substitutes are prebiotic substances that can induce the selective growth of probiotic microorganisms. Thus, chocolate that contains a combination of prebiotic substances and probiotic microorganisms is called synbiotic chocolate [4, 11].

The synbiotic chocolate can be divided into two subsets, called complementary synbiotic and synergistic synbiotic. A complementary synbiotic comprehends a product containing both probiotic and prebiotic, working independently to achieve one or more health benefits. The combination might not have solid evidence of synergistic function, but they provide health benefits separately. On the other hand, a synergistic synbiotic is composed of a probiotic microorganism and a specific prebiotic that have evidence of supporting the growth or activity of that specific microorganism. Although the substrate might also enrich other beneficial members of the gastrointestinal microbiota, its main target is to enhance the health benefits delivered when compared with probiotic and prebiotic separate effects due to the synergistic action [11].

The synbiotic chocolate production also involves the probiotic inoculation step besides the other traditional stages of chocolate processing, but instead of sugar, the prebiotic and other sugar-replacer ingredients are added, usually into the blending step with the other main ingredients. However, it is important to highlight that sugar replacement can significantly impact the quality of chocolate, including particle size, flow behavior, appearance, texture, melting profile, and moisture content. Thus, the development of synbiotic sugar-free and reduced-sugar chocolates with desirable physical and chemical properties for the food industry is currently a significant opportunity for scientists [4].

Therefore, considering the growing literature involving this theme, the objective of this protocol is to provide up-to-date and detailed information for the production of different types of probiotic and synbiotic chocolate.

2 Material

2.1 Chocolate Formulation

2.1.1 Probiotic Chocolate

Besides the inoculation of probiotic strains described in the **topic 2.2**, milk chocolate can be produced with 10.4% cocoa liquor, 18.9% cocoa butter, 41.5% sucrose powder, 25.4% milk powder (with 25% fat), 0.5% soy lecithin, and 0.06% ethyl vanillin [2]; dark chocolate can be produced with 35.9% cocoa liquor, 5% cocoa butter, 58.8% sucrose powder, and 0.5% soy lecithin [12]; and white chocolate can be produced with 44.5% sucrose powder, 30% cocoa butter, 16% powdered milk, 9% skimmed milk, 0.3% soy lecithin, and 0.2% PGPR [3].

2.1.2 Synbiotic Chocolate

Synbiotic chocolate comprises basically the sugar-free and reduced-sugar options (*see* **Notes 1**). Its formulation follows the same steps for each type of chocolate mentioned in **topic 2.1.1**, except that the sucrose content can be partially or totally replaced (*see* **Note 2**). Thus, besides the inoculation of probiotic strains described in the **topic 2.2**, a synbiotic sugar-free white chocolate, for example, can be produced with 44.5% maltitol, 30% cocoa butter, 16% powdered milk, 9% skimmed milk, 0.3% soy lecithin, and 0.2% PGPR. A variety of sweeteners and bulking agents used in chocolate formulation (*see* **Notes 3 and 4**) are summarized in Table 1 [3].

2.2 Probiotic Strains

Several probiotic strains are available for use as DVS inoculums in chocolate production (*see* **Notes 5 and 6**). The main cultures already used in probiotic chocolate and cell viability after prolonged storage are summarized in Table 2, while the main global exporter suppliers of DVS probiotic strains are provided in Table 3. The cell amount to be added into the chocolate varies between different strains in order to ensure the health benefits (*see* **Note 7**) [14–16].

2.3 Equipment for Chocolate Production

The necessary equipment for a pilot-scale probiotic and synbiotic chocolate production includes:

- A mixer with heating such as the planetary Vena mixer BM 30/20 (NV Machinery Verhoest, Izegem, Belgium);
- A pilot-scale-roll refiner such as the Exakt 80S 3-roll refiner (Exakt Apparatebau, Norderstedt, Germany);
- A chocolate conching machine such as the Buhler Elk'Olino conche (Richard Frisse GmbH, Bad Salzuflen, Germany);
- A chocolate-tempering machine such as the T5 (Pomati, Codogno, Italy);
- A chocolate vibration table such as the ZDT-02 (Food Machinery Service Co. Ltd., Nanquim, Jiangsu, China);
- A refrigerator (ranging at least from 0 to 20 °C);
- Plastic shapes for molding chocolate in the preferable dimension;
- A chocolate bar wrapping machine such as the Sleek 40 (Valtara, Schio, Italy) (optional), including the preferable packaging material (*see* **Note 8)** [21–23].

2.4 Chocolate Probiotic Viability Analysis

2.4.1 MRS Agar Formulation (See **Note 9**)

- Protease peptone (10 g/L);
- HM peptone B – the equivalent of beef extract (10 g/L);
- Yeast extract (5 g/L);
- Dextrose (20 g/L);
- Dipotassium phosphate (2 g/L);

Table 1
Sugar replacers and bulking agents in different synbiotic sugar-free types of chocolate and probiotic availability in the final product

Substitute	Replacement of the total sucrose content (%)	Type of chocolate	Probiotic strain	Inoculation dose (CFU/g)	Temperature and time of storage	Probiotic viability after storage	References
DVS							
Maltitol and inulin with DP > 23 and DP < 10	100 and 23.2	White	*L. paracasei* Lpc-37 ATCC SD5275 and *L. acidophilus* LA-14 ATCC SD5212	10^9	13–15 °C, 90 days	10^6–10^8 for both inulin types	[3]
Maltitol and inulin	68.7 and 31.3	Milk	*L. acidophilus* and *L. paracasei*	10^9	13–15 °C, 90 days	10^6	[13]
Microencapsulation							
Polydextrose and inulin	68.1 and 31.3	Dark	*L. plantarum* (299v) and *L. acidophilus* La3 (DSMZ 17742)	10^{13}	11 °C, ND	$< 10^8$	[6]
Isomalt and stevia	100 and 0.08	Dark	*L. plantarum* (299v) and *L. acidophilus* La3 (DSMZ 17742)	10^{13}	11 °C, ND	$< 10^8$	[6]
Isomalt and stevia	104.3 and 0.09	Milk	*L. plantarum* (299v) and *L. acidophilus* La3 (DSMZ 17742)	10^{13}	11 °C, ND	$>10^7$	[7]

DP Degree of polymerization, *ND* Not described

Table 2
Probiotic enrichment in different types of chocolate and its cell viability after storage

Probiotic strain	Inoculation dose (CFU/g)	Inoculation temperature (°C)	Type of chocolate	Temperature and time of storage	Probiotic viability after storage (CFU/g)	References
DVS						
L. acidophilus NCFM® and *B. lactis* HN019	10^8	30–32	Milk and dark	4 and 20 °C, 180 days	10^7–10^8	[17]
L. paracasei Lpc-37 ATCC SD5275 and *L. acidophilus* LA-14 ATCC SD5212	10^9	35	White	13–15 °C, 90 days	10^8–10^9	[3]
	10^9	35	Milk	13–15 °C, 90 days	10^6	[13]
Lacticaseibacillus rhamnosus, *L. paracasei* F19, *Lacticaseibacillus casei* DG and *Limosilactobacillus reuteri* DSM 17938	10^8–10^9	40	Dark	18 °C, 90 days	10^8, except *L. reuteri* (< 10^6)	[18]
L. acidophilus NCFM, *L. rhamnosus* HN001 and, *B. lactis* HN019	10^6–10^7	35 and 40	Milk	20 °C, 180 days	*L. rhamnosus* and *L. acidophilus* increased to 10^9 after 90 days. *B. lactis* decreased to <10^6 after 60 days	[2]
L. acidophilus NCFM and *B. lactis* HN019	10^8–10^{10}	30	Milk and dark	15 °C and fluctuating temperature (15–30 °C), 14 months	All strains remained >10^6 at 15 °C Almost all remained >10^6 at fluctuating temperature	[19]
Microencapsulation						
L. acidophilus (LA-5), *L. rhamnosus* (LGG), *Lactobacillus sanfranciscensis*, *Lactiplantibacillus plantarum*, *L. casei* 431, *Bifidobacterium*	10^{12}	45	Dark	4 and 25 °C, 180 days	> 10^7 after 180 days at 4 °C > 10^7 after 120 days at 25 °C	[8]

animalis subspp. *lactis* (BB-12), and *Streptococcus thermophilus*						
L. plantarum 564 and *L. plantarum* 299v	10^8	40	Dark	20 °C, 360 days	10^6 in 180 days. After that, decreased until 10^5	[20]
Immobilization						
L. casei 01 and *L. acidophilus* (LA-5)	10^{10}	35	White, milk and dark	4 and 25 °C, 60 days	All strains remained between 10^6 and 10^8 after 60 days at 4 °C and between 10^5–10^6 after 10 days at 25 °C	[21]

DVS Direct Vac Set inoculum

Table 3
Main global exporter suppliers of DVS probiotic strains

DVS supplier	Probiotic strains available[a]	Corporate Headquarter
Crh Hansen	*L. rhamnosus* (GR-1, LGG, DSM33560), *L. acidophilus* (DDS-1, LA-5, UALa-01), *L. paracasei* (CASEI 431, F-19, UALpc-04), *L. plantarum* (UALp-05), *L. casei* (UALc-03), *L. reuteri* (RC-14, LRC, UALre-16), *B. lactis* (UAB1a-12), *B. animalis* subsp. *lactis* (BB-12), *S. thermophilus* (TH-4, UASt-09)	Horsholm, Denmark
Sacco system	*L. rhamnosus (*CRL 1505, IMC 501), *L. paracasei IMC 502*, *L. plantarum LPLDL*	Cadorago, Italy
Danisco	*L. acidophilus* NCFM, *L. paracasei* Lpc-37 *B. lactis* (HN019, Bi-07, Bl-04), *L. lactis subsp. lactis*, *S. thermophilus* TA040	Copenhage, Denmark
Synbio tech Inc.	*L. acidophilus* LA1063, *L. casei* LC122, L. *paracasei* LPC48, *L. plantarum* LP198, *L. rhamnosus* LRH09, *B. animalis* subsp. *lactis* BAL06, *S. thermophilua* ST37	Yangzhou and Taiwan, China

[a]Only the probiotic species already described in the literature as being used in chocolate production were considered

- Sodium acetate (5 g/L);
- Triammonium citrate (2 g/L);
- Manganese sulphate (0.05 g/L);
- Tween 80 (1.08 g/L);
- Agar (15 g/L);
- Magnesium sulphate (0.2 g/L);
- L-Cysteine (0.05 g/L) (only for *Bifidobacterium* viable cells count) [24].

2.4.2 M17 Agar Formulation (See **Note 9**)

- Pancreatic digest of casein (5.3 g/L);
- Soy peptone (5.3 g/L);
- Beef Extract (5.3 g/L);
- Yeast Extract (2.6 g/L);
- Ascorbic Acid (0.5 g/L);
- Magnesium Sulfate (0.3 g);
- Disodium-β-glycerophosphate (20 g/L);
- Agar (11.5 g/L);
- Sterile lactose solution (100 g/L) [25].

Reagents, Solvents, and Solutions

- Acetic acid;
- Sodium hydroxide;
- Peptone water solution;
- Distilled water [7, 24].

2.4.3 Laboratory Glassware and Equipment

- Water bath;
- Microwave oven (optional);
- Glass bottle (volume of 1000 mL);
- Propylene centrifuge tube (volume of 50 mL);
- Sterile petri dishes;
- Pipette (sterile tips of 1 mL);
- Laminar flow cabinet;
- Bacteriological oven/Incubator;
- pHmeter [24, 25].

2.5 Chocolate Physical Analysis

- A water activity analyzer such as the Aqualab (METER Group, Inc., Hopkins, U.S.A);
- A colorimeter such as the Chroma Meter CR-400 (Konica Minolta, Tokyo, Japan);
- A texture analyzer such as the texture analyzer Model TA.HD. plus (Texture Technologies, Hamilton, U.S.A);
- A Differential scanning calorimeter;
- A laser scattering particle size distribution analyzer such as the MasterSizer® (Malvern Instrument, Malvern, U.K);
- A rheometer;
- A crusher equipment;
- An ultrasonic bath.

3 Methods

3.1 Probiotic and Synbiotic Chocolate Production

All the steps are summarized in the flowchart presented in Fig. 1, as well as the four main equipment used are presented in Fig. 2.

1. *Mixing*: mix the melted fat (20% of the total cocoa butter present in the formulation) and the other main ingredients (cocoa liquor, powdered sugar or sugar substitutes, powdered milk) during 12–15 min until they become homogeneous at 40 °C in a mixer with heating [20];
2. *Refining*: transfer the chocolate mass to a pilot-scale-roll refiner to achieve a mean particle size of approximately 20–25 μm (*see* **Note 10**) [3];
3. *Conching*: transfer the chocolate mass to a chocolate conching machine to execute two steps (*see* **Note 11**):
   (a) *Dry conching*: performed for 45 min at 60 °C;
   (b) *Wet conching*: the remaining cocoa butter (80% of the total), soy lecithin, and flavoring (when present in the formulation) is added, and then this process is maintained during 5 h, 25 min at 60 °C;

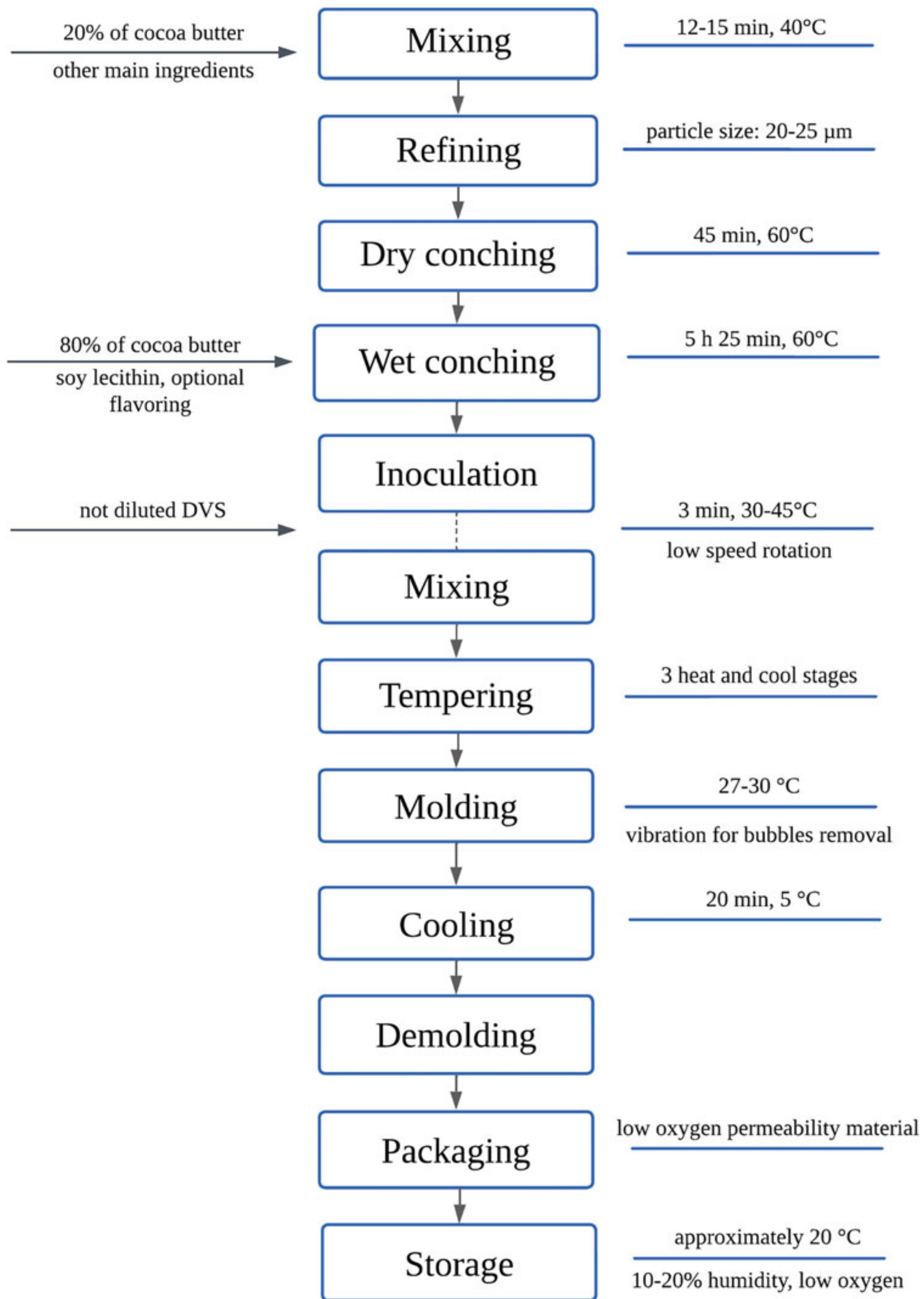

Fig. 1 Flowchart of probiotic and synbiotic chocolate production

Fig. 2 Chocolate processing during mixing, refining, conching and tempering steps. (Adapted from [31, 32])

4. *Probiotic inoculation and mixing*: transfer the chocolate mass again to the mixer with heating, where the content of the DVS is added directly into the melted product (*see* **Note 12**) with a temperature between 30 and 45 °C at a minimum number of rotations for 3 min [17];
5. *Tempering*: to provide cocoa butter crystallization in a preferred crystalline form (β-V), transfer the chocolate mass into a tempering machine to execute three steps:
   (a) Heating at 33–35 °C (white chocolate) or 45 °C (milk and dark chocolate) until melting [3, 13, 27];
   (b) Cooling between 25 and 28 °C for 5 min [13];
   (c) Heating again at 28–29 °C (milk chocolate) or 25 °C (white and dark chocolate) for conversion of any unstable crystals [3, 13, 27];
6. *Molding with vibration*: mold the final chocolate into plastic shapes and maintain it on the vibration table at 27–30 °C until the complete removal of air bubbles;
7. *Cooling*: cool the molded chocolate at 5 °C in a refrigerator for 20 min for complete solidification (*see* **Note 13**) [3, 28];
8. *Demolding and packaging*: demold the chocolate and package it in a specific equipment or manually [29];
9. *Storage*: store the packaged chocolate at 20 °C or other temperatures, if preferable (*see* Tables 1 and 2), with a range of humidity between 10 and 20% and preferable with low oxygen (*see* **Note 14**) [30].

3.2 Quality Analysis Related to Probiotic and Synbiotic Chocolate

3.2.1 Chocolate Probiotic Viability Analysis

Preparation of MRS Agar

1. Suspend all the components from the culture medium in 1000 mL distilled water in a glass bottle (*see* **Note 15**).
2. Check the final pH at 25 °C, that must be at 6.5 ± 0.2 (*see* **Note 16**);
3. Heat it in a microwave oven (*see* **Note 17**) or a water batch can be necessary to dissolve the medium completely;
4. Sterilize by autoclaving at 15lbs pressure (121 °C) for 15 min;
5. Cool the MRS agar (45–50 °C) before use, and if not used promptly store in refrigeration (2–8 °C) [25].

Preparation of M17 Agar

1. Suspend all the components from culture medium, with the exception of the lactose in 1000 mL distilled water in a glass bottle (*see* **Note 15**);
2. Heat it in a microwave oven (*see* **Note 17**) or a water batch to dissolve the medium completely;
3. Sterilize by autoclaving at 15lbs pressure (121 °C) for 15 min;
4. Cool the bottle until 50 °C to add 50 mL sterile lactose solution and mix;
5. If M17 agar is not used promptly, store it in refrigeration (2–8 °C) (*see* **Note 18**) [26].

Viable Cell Count

To investigate the probiotic viability in the chocolate formulation during storage, carry out the analysis described below in the same day that the chocolate was produced, and repeat it with the desirable frequency (for example on 0th, 30th, 60th, and 90th days of storage) (*see* **Note 19**):

1. Take approximately 25 ± 0.2 g of chocolate under aseptic conditions and mix it with 180 mL peptone water solution;
2. Melt the mixture in a water bath for 15 min at 40 °C;
3. Prepare a decimal dilution series;
4. Plate 1 mL of each dilution (triplicate) and add 15–25 mL of molten selective media (45–50 °C) in petri dishes, using the proper inoculation mode, which will depend on the probiotic strain included into the formulation (*see* **Note 20**);
5. Gently shake the plate to mix the inoculum in the selective media;
6. Let the media solidify;
7. Incubate petri dishes under adequate conditions, which will depend on the probiotic strain included into the formulation (*see* **Note 21**);
8. After the incubation period, make the colony count. Register the results as CFU/g [7].

3.2.2 Chocolate Physical Analysis

The physical analysis of chocolates is one of the most important quality parameters for the food industry. From the results of these analyses, we can predict, e.g., the shelf life of the product and the melt in the mouth. Below we describe the procedures of the main physical analyzes performed on chocolates.

Water Activity

1. *Preparing the sample*: Grind the chocolate in a crusher.
2. *Reading on the device*: Transfer the 2 g of chocolate to a water activity analyzer, and read at 25 °C [33].

Color Measurement

1. *Preparing the sample*: Grind the chocolate in a crusher.
2. *Reading on the device*: Transfer the 5 g of chocolate to a Petri plate and place a white sheet under it. Then, the color parameters such as L: brightness, a: ± red-green, and b: ± yellow-blue can be measured using a colorimeter. In addition, the chroma (C*) and whiteness index (WI) parameters can be calculated using Eqs. 1 and 2. It is advisable to carry out this analysis if any substance in the chocolate formulation changes the color of the final product [33].

$$C^* = \sqrt{a^2 + b^2} \quad (1)$$

$$WI = 100 - \sqrt{(100 - L)^2 + (a)^2 + (b)^2} \quad (2)$$

Texture Analysis

1. *Preparing the sample*: Cut the chocolate into approximately 1 cm^2 square.
2. *Performing the analysis*: As a texture parameter, hardness is the most important to determine in chocolates. Place the sample in a texture analyzer and operate using a 500 N load cell with a pre-test speed of 1 mm s^{-1} and a firing force of 0.1 N. Pre-test, test, and post-test speeds applied during textural measurement can be adjusted from 1 mm s^{-1}, 1 mm s^{-1}, and 10 mm s^{-1}, respectively. The hardness values of each sample must be measured at least 7 times [3].

Fusion Properties

1. *Preparing the sample*: Grind the chocolate in a crusher.
2. *Performing the analysis*: Transfer the 15 mg of chocolate to aluminum crucibles and analyze them in a differential scanning calorimeter (DSC) at 0–70 °C under a heating rate of 5 °C min^{-1} and nitrogen flow of 50 mL min^{-1} [34].

Particle-Size Distribution

1. *Preparing the sample*: Grind the chocolate in a crusher.
2. *Performing the analysis*: Disperse approximately 0.20 g of chocolate in vegetable oil (refractive index, RI = 1.45) at room temperature (20 ± 2 °C) until an obscuration of 0.2 is obtained. Then, keep the sample in an ultrasonic bath for 2 min to ensure that the particles are freely dispersed. Finally, read on a laser scattering particle size distribution analyzer [33, 35].

Rheological Measurements

1. *Preparing the sample*: Melt the chocolate in an oven at 50 °C for 75 min.
2. *Performing the analysis*: Transfer the chocolate to a rheometer and shear at a rate of 5.0 s^{-1} for 10 min at 40 °C before the measurement cycles start. Measure the shear stress at 40 °C with increasing shear rate from 0.5 to 60 s^{-1} (ramp up) in 120 s

and then decrease the shear rate from 60 to 0.5 s^{-1} (ramp down). On each ramp, 50 measurements must be taken. This measurement cycle must be repeated 30 consecutive times until thixotropy is eliminated from the samples. The measurement data can be applied to the Casson Model (Model recommended for chocolates) (Eq. 3) to determine related rheological parameters such as yield stress, Casson yield stress, and Casson viscosity [33, 35, 36].

$$\sqrt{\tau} = \sqrt{\tau_{CA}} + \sqrt{\mu * CA} * \sqrt{\gamma} \quad (3)$$

Where, τ: yield stress; τ_{CA}: Casson yield stress; $\mu * CA$: Casson viscosity, and γ: shear rate.

4 Notes

1. Besides sugar-free and reduced-sugar chocolates, low-fat products have also been produced, using defatted cocoa derivatives or fat replacers with prebiotic functionality such as inulin, β-glucan, xanthan gum, and guar gum. However, most studies have discussed the impact of fat reduction or fat replacement on the quality attributes of chocolate and do not discuss the nutritional effect. In addition, there are no studies describing the production of low-fat chocolate with probiotic or synbiotic functionality. Thus, investigating the effect of probiotic fat-reduced chocolate on health is an excellent opportunity for a study topic [4];
2. Although regulations can change slightly between countries, in the case of reduced-sugar chocolates, the "light" claim can be used in the product label when there is approximately 25% of sugar reduction when compared to the regular version [37];
3. Although polyols can present a prebiotic functionality, high concentrations can cause abdominal discomforts. Thus, care must be taken when formulating chocolate with this type of ingredient [38];
4. It is important to notice that sweeteners and bulk agents can replace the total content of sucrose by 100% or a little bit more, which can slightly alter the proportion of other ingredients into the chocolate formulation;
5. Several strains are already considered probiotic and are cited in the literature. Besides them, FAO states Guidelines for the Evaluation of Probiotics in Food [39] when the potential of some strains still has to be investigated before being added to the food product;
6. DVS is widely used for probiotic chocolate production. However, in some cases, other approaches, such as microencapsulation (*see* Chap. 14), can ensure greater protection of probiotics;

7. To ensure the health benefits provided by probiotic strains into the chocolate formulation, 10^1 to 10^2 CFU/g above the desirable amount can be added, considering the potential cell losses during chocolate processing and storage [7];
8. Most dairy probiotics and other products are stored and sold on the market in plastic packaging with high oxygen permeability, this poses a serious problem to the growth and survival of the probiotic. The use of plastic films with high oxygen barrier properties and active packages with oxygen absorbers or glass containers can be a solution [28];
9. After opening, the product should be properly stored dry, after tightly capping the bottle due to the hygroscopic nature of the product [40];
10. Human taste buds allow for detecting particles larger than 25 μm. Thus, a lower particle size in chocolate products is desirable. In 3-roller machines, the chocolate mass must be treated two or three times to obtain this size, while in 5-roll refiners it can be achieved in a single process. Rotation speed and temperature can be adjusted to approximately 0.75 m/s and 30 °C for the first roll, 1.25 m/s and 35 °C for the second, 1.80 m/s, 40 °C for the third, 2.45 m/s and 45 °C, and 3.70 m/s and 40 °C for the fifth [41];
11. The literature describes a range of temperatures employed in the conching process of different types of chocolate. Overall, milk and white chocolate conching temperatures range between 40 and 70 °C. Temperatures above 70 °C cannot be exceeded in those cases once it provides the denaturation of milk proteins into the formulation. But the lack of milk solids in dark chocolate formulation allows it to be heated between 40 and 80 °C. The longer the time and temperature are applied, the greater the *Maillard* reaction. However, temperatures above 60 °C tend to reduce soy lecithin efficiency. Thus, it was suggested to establish a temperature of approximately 60 °C for all types of chocolate [40, 42];
12. Additives and probiotic powders are generally resuspended in some liquid ingredients before being added to the product formulation. However, resuspension of DVS probiotic cells in UHT milk before adding to chocolate has been shown to cause significant viability losses for *L. rhamnosus* GG, *L. paracasei* F19, *L. paracasei* DG, and *L. reuteri* DSM17938. This fact is probably justified due to the induction of an early reactivation of freeze-dried cells when resuspended, becoming more sensitive to stress conditions during the final stages of chocolate production. Thus, an anabiotic state of cells is preserved when added directly to chocolate, which has been shown to ensure greater survival [18];

13. The different chocolate shapes (chocolate bars, bon bons, and other filling products) are made with the same ingredients and production steps. But in the case of filled chocolates, the melted chocolate must be molded into semi-sphere shapes, an additional step must be included, which consists of filling the molded chocolate, adding melted chocolate above the filling or joining a second semi-sphere chocolate to the first. Then the final product can be cooled [43];
14. Besides a proper packaging material, vacuum storage was proven to be better than nitrogen or air when it comes to probiotic viability into chocolate. Also, its survival is inversely related to storage temperature [30];
15. Currently it is possible to acquire the commercial powder containing all the necessary ingredients of the selective media. In those cases, it is necessary only to resuspend the mixture powder in distilled water, and proceed with sterilization.
16. Occasionally, sterilization may cause the pH to fall outside of the specified pH limits. In these rare cases, pH adjustment using acetic acid or sodium hydroxide is recommended [25];
17. Heating in a microwave oven may be uneven across the total volume of the medium. This can be prevented if you slowly shake the flask when it starts to boil and reheat further until the medium is totally dissolved [44];
18. The addition of lactose must be done only in the selective media that will be plated immediately. The part of the agar that will be stored for future plating can't contain lactose, because it can't be melted again. Thus, it must be discarded.
19. The inoculation procedure requires skilled labor and must be conducted aseptically in an adequate laboratory (in a laminar flow cabinet) and respecting the good laboratory practices to avoid contamination [45];
20. It is not necessary to use a highly selective media when counting pure cultures added into a food formulation. Thus, for *Lactobacillus*, *Lacticaseibacillus*, and *Limosilactobacillus*, it is possible to use a MRS agar in a pour plate technique. For *Bifidobacterium*, the MRS agar must be supplemented with 0.05% cysteine and inoculated with pour plate technique. For *Streptococcus*, the M17 agar is recommended with a spread plate procedure [45–47]. For more details about colony counting and selective media, check the Chap. 25 called "probiotics" present in the "Handbook of Dairy Food Analysis" [48];
21. *Lactobacillus*, *Lacticaseibacillus*, *Limosilactobacillus*, and *Bifidobacterium* must be incubated at 37 °C for 48 h, while the *Streptococcus* must be incubated at 42 °C for 48 h, all under anaerobic conditions [7, 49].

Acknowledgments

The authors are grateful to CAPES (Coordination of Improvement of Higher Education Personnel) for the scholarship (Finance code 001).

References

1. Selvasekaran P, Chidambaram R (2021) Advances in formulation for the production of low-fat, fat-free, low-sugar, and sugar-free chocolates: an overview of the past decade. Trends Food Sci Technol 113:315–334
2. Zarić DB, Bulatović ML, Rakin MB, Krunić TŽ, Lončarević IS, Pajin BS (2016) Functional, rheological and sensory properties of probiotic milk chocolate produced in a ball mill. RSC Adv 6(17):13934–13941
3. Konar N, Palabiyik I, Toker OS, Polat DG, Kelleci E, Pirouzian HR, Akcicekc A, Sagdic O (2018) Conventional and sugar-free probiotic white chocolate: effect of inulin DP on various quality properties and viability of probiotics. J Funct Foods 43:206–213
4. Muhammad DRA, Rahayu ES, Fibri DLN (2021) Revisiting the development of probiotic-based functional chocolates. Rev Agric Sci 9:233–248
5. Konar N, Toker OS, Oba S, Sagdic O (2016) Improving functionality of chocolate: a review on probiotic, prebiotic, and/or synbiotic characteristics. Trends Food Sci Technol 49:35–44
6. Gómez-Fernández AR, Faccinetto-Beltrán P, Orozco-Sánchez NE, Pérez-Carrillo E, Santacruz A, Jacobo-Velázquez DA (2021a) Physicochemical properties and sensory acceptability of sugar-free dark chocolate formulations added with probiotics. Revista Mexicana de Ingeniería Química 20(2):697–709
7. Gómez-Fernández AR, Faccinetto-Beltrán P, Orozco-Sánchez NE, Pérez-Carrillo E, Marín-Obispo LM, Hernández-Brenes C, Santacruz A, Jacobo-Velázquez DA (2021b) Sugar-free milk chocolate as a carrier of omega-3 polyunsaturated fatty acids and probiotics: a potential functional food for the diabetic population. Foods 10(8):1866
8. Hossain MN, Ranadheera CS, Fang Z, Ajlouni S (2021) Impact of encapsulating probiotics with cocoa powder on the viability of probiotics during chocolate processing, storage, and in vitro gastrointestinal digestion. J Food Sci 86(5):1629–1641
9. Reale A, Renzo TD, Coppola R (2019) Factors affecting viability of selected probiotics during cheese-making of pasta filata dairy products obtained by direct-to-vat inoculation system. LWT 116:108476
10. Rodrigues MG, Sartori JDO, Amaral DJC, Oliveira PDPD, Rabito EI (2020) Picolés hiperprotéicos e hipercalóricos são uma boa alternativa dietética no tratamento de pacientes em terapia antineoplásica ambulatorial. Nutrición clínica Dietética Hospitalaria 40:25–32
11. Swanson KS, Gibson GR, Hutkins R, Reimer RA, Rei G, Verbeke K, Scott KP, Holscher HD, Azad MB, Delzenne NM, Sanders ME (2020) The international scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of synbiotics. Nat Rev Gastroenterol Hepatol 17(11): 687–701
12. Afoakwa EO, Paterson A, Fowler M (2008) Effects of particle size distribution and composition on rheological properties of dark chocolate. Eur Food Res Technol 226(6): 1259–1268
13. Toker OS, Polat DG, Gulfidan OG, Konar N, Palabiyik İ, Akcicek A, Poyrazoglu ES, Sagdic O (2017) Stability of lactic acid bacteria in synbiotic sugared and sugar-free milk chocolates. Int J Food Prop 20:1354–1365
14. Oliveira D, Antúnez L, Giménez A, Castura JC, Deliza R, Ares G (2015) Sugar reduction in probiotic chocolate-flavored milk: impact on dynamic sensory profile and liking. Food Res Int 75:148–156
15. Kobus-Cisowska J, Szymanowska D, Maciejewska P, Szczepaniak O, Kmiecik D, Gramza-Michałowska A, Kulczynski B, Cielecka-Piontek J (2019) Enriching novel dark chocolate with Bacillus coagulans as a way to provide beneficial nutrients. Food Funct 10(2):997–1006
16. Hossain MN, Ranadheera CS, Fang Z, Ajlouni S (2020) Healthy chocolate enriched with probiotics: a review. Food Sci Technol 41:531–543
17. Lalicic-Petronijevic J, Popov-Raljić J, Lazić V, Pezo L, Nedović V (2017) Synergistic effect of three encapsulated strains of probiotic bacteria on quality parameters of chocolates with different composition. J Funct Foods 38:329–337

18. Succi M, Tremonte P, Pannella G, Tipaldi L, Cozzolino A, Coppola R, Sorrentino E (2017) Survival of commercial probiotic strains in dark chocolate with high cocoa and phenols content during the storage and in a static in vitro digestion model. J Funct Foods 35:60–67
19. Klindt-Toldam S, Larsen SK, Saaby L, Olsen LR, Svenstrup G, Müllertz A, Knochel S, Heimdal H, Nielsen DS, Zielińska D (2016) Survival of *Lactobacillus acidophilus* NCFM® and *Bifidobacterium lactis* HN019 encapsulated in chocolate during in vitro simulated passage of the upper gastrointestinal tract. LWT 74:404–410
20. Mirkovic M, Seratlic S, Kilcawley K, Mannion D, Mirkovic N, Radulovic Z (2018) The sensory quality and volatile profile of dark chocolate enriched with encapsulated probiotic lactobacillus plantarum bacteria. Sensors 18(8):2570
21. Kemsawasd V, Chaikham P, Rattanasena P (2016) Survival of immobilized probiotics in chocolate during storage and with an in vitro gastrointestinal model. Food Biosci 16:37–43
22. Saputro AD, Walle VD, Caiquo BA, Hinneh M, Kluczykoff M, Dewettinck K (2019) Rheological behaviour and microstructural properties of dark chocolate produced by combination of a ball mill and a liquefier device as a small scale chocolate production system. LWT 100:10–19
23. Polinski S, Topka P, Tańska M, Kowalska S, Czaplicki S, Szydłowska-Czerniak A (2022) Impact of bioactive compounds of plant leaf powders in white chocolate production: changes in antioxidant properties during the technological processes. Antioxidants 11(4): 752
24. VT – Valtare Tech (2022) Sleek F40 wrapping machine. Retrieved from https://www.valtaratec.com/sleek-40-compact-flow-wrapping-machine/. Accessed 09 Aug 2022
25. Neogen (2002) Specification of MRS agar selective medium. Retrieved from https://www.neogen.com/categories/microbiology/mrs-agar/. Accessed 10 Aug 2022
26. BD (2022) Manual: MR17 agar and MR17 broth. Retrieved from https://legacy.bd.com/europe/regulatory/Assets/IFU/Difco_BBL/218561.pdf. Accessed 10 Aug 2022
27. Briggs JL, Wang T (2004) Influence of shearing and time on the rheological properties of milk chocolate during tempering. J Am Oil Chem Soc 81(2):117–121
28. Mizielinska M, Sobecka K, Jarosz M, Urbański D, Stobińska M, Łukawska B, Olchawa E, Bartkowiak A (2017) The influence of immobilization and packaging on the viability of probiotics stored at 25 C. World Sci News 77(2):124–143
29. Islam MZ, Masum AKM, Harun-ur-Rashid M (2022) Milk chocolate matrix as a carrier of novel lactobacillus acidophilus LDMB-01: physicochemical analysis, probiotic storage stability and in vitro gastrointestinal digestion. J Agric Food Res 7:100263
30. Haffner FB, Diab R, Pasc A (2016) Encapsulation of probiotics: insights into academic and industrial approaches. AIMS Mater Sci 3(1): 114–136
31. Hinneh M, Walle DV, Haeck J, Abotsi EE, Winne A, Saputro AD, Messens K, Durme JV, Afoakwa EO, Cooman L, Dewettinck K (2019) Applicability of the melanger for chocolate refining and Stephan mixer for conching as small-scale alternative chocolate production techniques. J Food Eng 253:59–71
32. SaintyCo (2022) Pharma process and packaging: chocolate tempering machine – the ultimate FAQ guide. Retrieved from https://www.saintytec.com/chocolate-tempering-machine/ . Accessed 10 Aug 2022
33. Konar N (2013) Influence of conching temperature and some bulk sweeteners on physical and rheological properties of prebiotic milk chocolate containing inulin. Eur Food Res Technol 236:135–143. https://doi.org/10.1007/s00217-012-1873-x
34. Razavizadeh BM, Tabrizi P (2021) Characterization of fortified compound milk chocolate with microcapsulated chia seed oil. LWT – Food Sci Technol 150:111993. https://doi.org/10.1016/J.LWT.2021.111993
35. Ohene EA, Paterson A, Fowler M (2008) Effects of particle size distribution and composition on rheological properties of dark chocolate. Eur Food Res Technol 226:1259–1268. https://doi.org/10.1007/s00217-007-0652-6
36. Bolenz S, Glöde L (2021) Technological and nutritional aspects of milk chocolate enriched with grape pomace products. Eur Food Res Technol 247:623–636. https://doi.org/10.1007/s00217-020-03651-4
37. Soares AF, Honorio AR, de Lima DCN, Tribst AA (2021) Sweet processed foods in Brazil: use of sugar and sweeteners, inclusion of sugar claims and impact on nutritional profile. Int J Food Sci Technol 56(9):4428–4433
38. Lenhart A, Chey WD (2017) A systematic review of the effects of polyols on gastrointestinal health and irritable bowel syndrome. Adv Nutr 8(4):587–596

39. FAO – Food and Agriculture Organization (2002) Guidelines for the evaluation of probiotics in food. Joint FAO/WHO, Ontario
40. HiMedia (2022). Technical data: Lactobacillus MRS agar. Retrieved from https://himedialabs.com/TD/M641.pdf. Acessed 10 Aug 2022
41. CC – Cocoa and Chocolate (2022) Chocolate manufacturing: grinding method. Retrieved from https://www.cacaochocolade.nl/main.php?p=inhoud&h=5. Accessed 29 June 2022
42. Cohen KDO, Jackix M (2009) Efeito do processo de conchagem nas características físicas e químicas do chocolate ao leite e produtos análogos. Embrapa Cerrados-Documentos (INFOTECA-E), Planaltina
43. Seçuk B, Seçim Y (2021) Development of chili pepper ganache filled chocolate in artisan chocolate production, determination of sensory and physicochemical characteristics. Food Sci Technol 42:1–9
44. Avantor (2022) MRS agar (ISO) 200mL: description/technique. Retrieved from http://www.reactivosparadiagnostico.com/customers/viewTDS.php?file=400587ZA_EN.pdf&id=3ab0574d437b632f4e2d4e7f5b5b79a03c80e85a7a206f1. Accessed 10 Aug 2022
45. Moradi M, Kousheh SA, Razavi R, Rasouli Y, Ghorbani M, Divsalar E, Tajik H, Guimarães JT, Ibrahim SA (2021) Review of microbiological methods for testing protein and carbohydrate-based antimicrobial food packaging. Trends Food Sci Technol 111:595–609
46. Verruck S, Barretta C, Miotto M, Canella MHM, de Liz GR, Maran BM, Garcia SG, Silveira SM, Vieira CRW, Cruz AG, Prudencio ES (2020) Evaluation of the interaction between microencapsulated *Bifidobacterium* BB-12 added in goat's milk frozen yogurt and Escherichia coli in the large intestine. Food Res Int 127:108690
47. Korcz E, Varga L, Kerényi Z (2021) Relationship between total cell counts and exopolysaccharide production of Streptococcus thermophilus T9 in reconstituted skim milk. LWT 148:111775
48. Nollet LML, Toldrá F (2021) Handbook of dairy foods analysis. CRC Press, Boca Ratón
49. Ozturk B, Elvan M, Ozer M, Harsa ST (2021) Effect of different microencapsulating materials on the viability of *S. thermophilus* CCM4757 incorporated into dark and milk chocolates. Food Biosci 44:101413

Chapter 14

Microencapsulation of Probiotics

Callebe Camelo-Silva, Lais Leite Figueredo, Vanessa Cortina Zanetti, Alan Ambrosi, Marco Di Luccio, and Silvani Verruck

Abstract

Probiotics are susceptible to factors such as stomach acid, enzymes, and bile salts. Also, when incorporated into food matrices, intrinsic or processing factors like low pH, high water activity, or high cooking temperatures can negatively affect the viability of microorganisms. Encapsulation technology can ensure the safe delivery of probiotics to the gut and better survival during processing and storage. Several techniques are used to protect probiotics, for example, emulsion, extrusion, spray-drying, freeze-drying, liposome, electrospinning, and others. Here, we describe in detail the main methods of encapsulation of probiotics, including emulsion, extrusion, and spray-drying techniques.

Key words Probiotic, Encapsulation, Emulsion, Extrusion, Spray-drying

1 Introduction

The consumption of probiotic products has increased exponentially due to the range of benefits these microorganisms can offer to human health. However, it is still a challenge to ensure the viability of probiotics to the consumer, as they have a noticeable loss of viability after passing through the digestive tract. In addition, when incorporated into commercial products, intrinsic or processing factors such as low pH, high water activity, or high cooking temperatures can negatively affect the viability of microorganisms [1].

Microencapsulation emerges as an alternative to circumvent these limitations. This technique is based on trapping probiotics within an encapsulating matrix, ensuring safe delivery to the intestine at appropriate therapeutic levels to provide human health benefits [2]. Several microencapsulation techniques can be used to encapsulate probiotics (Table 1). However, emulsion, extrusion, and spray-drying techniques occupy a prominent place, considering

Adriano Gomes da Cruz et al. (eds.), *Probiotic Foods and Beverages: Technologies and Protocols*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-3187-4_14,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2023

Table 1
Encapsulation techniques used to microencapsulate probiotics

Encapsulation technique	Probiotic strain	Wall materials	Encapsulation yield (%)	References
Emulsion	*Lactiplantibacillus plantarum* (MT, ZH593)	Alginate	27–82	[7]
Extrusion	*Limosilactobacillus reuteri* (DSM 20016)	Alginate and (tamarind gum or mutamba mucilage or cassia tora gum or psyllium mucilage or konjac gum)	93–97	[8]
Spray-drying	*Bifidobacterium animalis* subsp. *lactis* BB-12	Full-fat goat's milk and/or prebiotics (inulin and/or oligofructose)	94–97	[9]
Freeze-drying	*Lactobacillus acidophilus* (La-05), *Lacticaseibacillus casei* (Lc-01)	Microalgae *Spirulina platensis*, *Chlorella vulgaris*, *Scenedesmus quadricauda*, and *Lagerheimia longiseta*	80–92	[10]
Supercritical	*Bifidobacterium animalis subsp. lactis* BB-12, *Bifidobacterium longum* BB-46	Poly-(vinylpyrrolidone)-poly-(vinylacetate-co-crotonic acid)	Not shown	[11]
Liposome	*Lacticaseibacillus rhamnosus* (ATCC 10754)	Lecithin and (chitosan or gelatin)	81–87	[12]
Electrospinning	*Lacticaseibacillus rhamnosus* 1.0320	Pectin and poly (vinyl alcohol)	Not shown	[13]
Microfluidics	*Saccharomyces cerevisiae* (PDC1-GFP)	Alginate	Not shown	[14]
Layer-by-layer	*Ligilactobacillus salivarius* Li01 (Li01)	Chitosan and alginate	Not shown	[15]
Fluidized bed	*Lactobacillus acidophilus* (PTCC 1643)	Xanthan, alginate, chitosan, and gellan	35–78	[16]
3D printing	*Bifidobacterium lactis* (HOWARU® Bifidous) *Lactobacillus acidophilus* (HOWARU® Dophilus)	Alginate and gelatin	Not shown	[17]

their low cost, simplicity of handling, and the possibility of producing large-scale microcapsules. Thus, throughout this chapter, we will address only these most used techniques.

The encapsulating matrix can be formed using different wall materials, also known as carriers. Sodium alginate has been widely used due to its low cost, biocompatibility, food grade, and targeted delivery of probiotics (soluble in basic medium, for example, in the intestine) [3]. Wall materials such as chitosan, gelatin, milk proteins, pectin, carrageenan, prebiotics, and different types of starch have also occupied a prominent place for the microencapsulation of probiotic strains. The criteria for choosing a suitable encapsulating agent are mainly based on its physicochemical properties (molecular mass, solubility, glass transition temperatures, crystallinity, film formation, and emulsifying properties). A good wall material must also be easy to handle during the encapsulation process. In addition, it cannot react or injure the probiotic strain during the encapsulation and storage process and, finally, it must meet the solubility properties of the microcapsule by releasing the probiotics at the site of action [1]. To describe the methodology of this chapter, we will consider alginate (ALG) and whey proteins (WPI) as encapsulating agents and the strain of *Lacticaseibacillus rhamnosus* GG as active material. Alginate was chosen because it is necessary to use a hydrocolloid for the crosslinking process in the emulsion and extrusion methods. In addition, it is considered GRAS (Generally Recognized as Safe) and low cost. However, other wall materials have been widely used [4–6].

2 Material

For the production of microcapsules, the following materials are needed:

- Freeze-dried probiotic cells;
- De Man Rogosa and Sharpe (MRS) broth;
- Glycerol.
- Bacteriological oven;
- Centrifuge;
- Saline solution;
- Soybean oil;
- Alginate (ALG);
- Whey proteins (WPI);
- Calcium carbonate;

- Acid organic;
- Span 80;
- Calcium chloride;
- Spray-drier.

3 Methods

3.1 Preparation of Probiotic Suspension

To obtain the stock solution, freeze-dried probiotic cells can be rehydrated in sterile skim milk (25 g L^{-1}) or with De Man Rogosa and Sharpe (MRS) broth added with glycerol (20 g L^{-1}) and stored in sterile Falcon vials at -20 ± 2 °C [18] (*see* **Note 1**). Then, the stock solution is added to sterile MRS broth and incubated (37 ± 1 °C for 48 h) to reach the stationary phase (*see* **Note 2**). After the incubation time, the probiotic cells are harvested by centrifugation ($1000 \times g$) for 10 min at a temperature of 25 ± 1 °C and washed twice with saline solution (0.9 g 100 mL^{-1}). Cell pellets should be kept at 4 ± 1 °C until encapsulation procedure.

3.2 Encapsulation of Probiotics by Emulsion

The emulsion technique consists of mixing two immiscible phases, called the dispersed or discontinuous phase, and the oily or continuous phase [1]. In this method, ALG gelation can be performed internally or externally (Fig. 1). In internal gelation, the alginate is previously solubilized with calcium carbonate, and then an aliquot of organic acid is added to the mixture after emulsification to

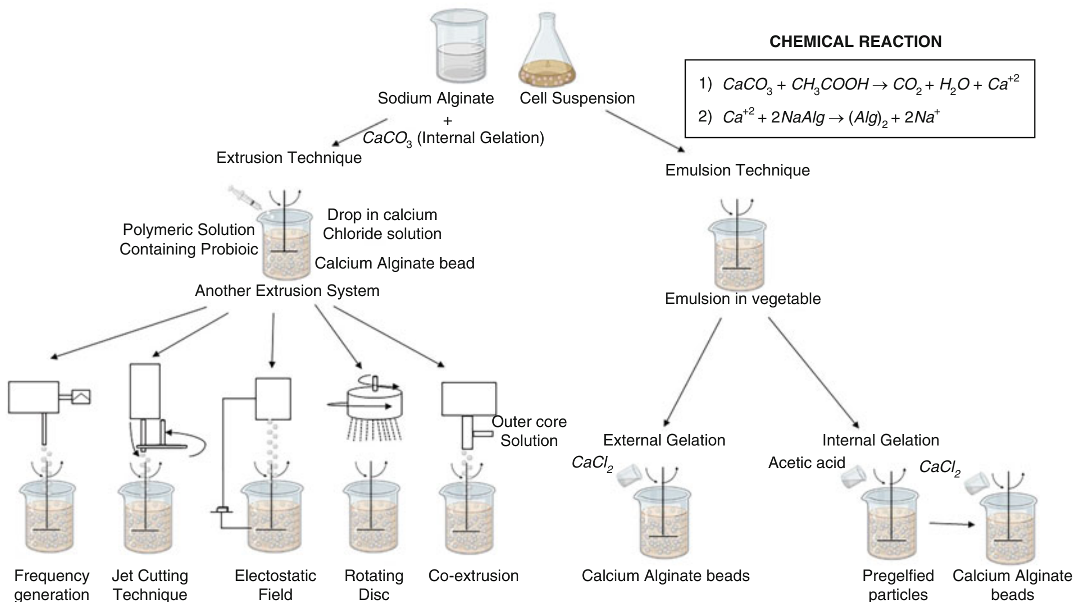

Fig. 1. Extrusion and emulsion technologies [19]. (Adapted from [19])

promote gelation. As the organic acid enters the aqueous phase, it interacts with calcium carbonate, releasing calcium ions and carbonic acid. The calcium ions react with the alginate through complexation with the carboxylic groups of the polymer, forming the "egg box model" structure [19]. On the other hand, in external gelation, the complexation reaction of the carboxylic groups of ALG occurs through contact with a solution of calcium chloride.

1. Preparing the dispersed phase: Mix 5% (w/v) of WPI in 100 mL of sterile distilled water under stirring at 400 rpm. Then, gently add 1% (w/v) of ALG (*see* **Note 3**), and leave under stirring until the alginate is entirely homogenized.
2. Addition of cell biomass: Aseptically, an aliquot (~ 9 log CFU mL^{-1}) of the probiotic biomass should be added to the dispersed phase and then homogenized at 400 rpm for 5 min. It is recommended to add a biomass content that reaches a viable cell count of around 9 to 10 log CFU g^{-1} (*see* **Note 4**).
3. Preparing the continuous phase: Add 300 mL of soybean oil to a beaker. Add 3% (v/v) of an emulsifying agent (Span 80) in the same container and leave it under stirring at 400 rpm until complete homogenization (*see* **Note 5**).
4. Mixing the two phases: In a beaker, mix the dispersed and continuous phases and leave under stirring at 400 rpm for 20 min or until the complete formation of the emulsion (*see* **Note 6**).
5. ALG cross-linking process: While stirring, add an aliquot of a 1.5% (w/v) calcium chloride solution to form the gelled microcapsules (*see* **Note 7**). Then, turn off the agitation and add 200 mL of sterile distilled water to attract the microcapsules to the aqueous phase.
6. Collecting the microcapsules: Discard the emulsion supernatant, collect the microcapsules by vacuum filtration, and keep them at 4 °C until drying.
7. Drying of microcapsules: Gelled microcapsules can be dried in a spray-dryer, freeze-dryer, or fluidized bed dryer (*see* **Note 8**). After drying, the microcapsules can be packed in airtight packaging and kept at room temperature until use (*see* **Note 9**).

3.3 Encapsulation of Probiotics by Extrusion

The extrusion technique (Fig. 1) involves mixing the cellular biomass of the probiotic with the polymeric solution (ALG and WPI) and then forming droplets by passing the solution through a nozzle or atomizing nozzle [20].

1. Preparing the dispersed phase: Mix 5% (w/v) f WPI in 100 mL of sterile distilled water under stirring at 400 rpm. Then, gently add 1% (w/v) of ALG (*see* **Note 3**), and leave under stirring until the alginate is entirely homogenized.

2. Addition of cell biomass: Aseptically, an aliquot (~ 9 log CFU mL^{-1}) of the probiotic biomass should be added to the dispersed phase and then homogenized at 400 rpm for 5 min. It is recommended to add an aliquot with a viable cell count of around 8 to 9 log CFU g^{-1} (*see* **Note 4**). It is worth emphasizing that the dispersed phase containing the hydrocolloid must be prepared just before use.
3. Forming the gelled microcapsules: Once the feed solution (FS) (polymer solution + probiotic) is prepared, the FS is dripped into a 1.5% (w/v) calcium chloride (*see* **Note 7**) gelling solution under stirring at 200 rpm. The dripping of the FS into the gelling solution is carried out using an atomizing nozzle. In this case, the FS is pumped by a peristaltic pump, and the droplets are quickly transformed into solid particles through the complexation of ALG with calcium ions. Another simplified form can be used, for example, using a syringe to perform the drip (*see* **Note 10**). After the dripping step, it is interesting to leave the microcapsules to rest (~20 to 30 min) in the $CaCl_2$ solution to solidify the microcapsules completely. The formation of large particles and the low production rate are the main disadvantages of this technique for use in the food industry. However, to overcome this, the extrusion process can be combined with ultrasound, jet cutting, electrostatic field, and rotating disk (Fig. 1).
4. Collecting the gelled microcapsules: Collect the microcapsules by vacuum filtration and keep them at 4 °C until drying.
5. Drying of microcapsules: Gelled microcapsules can be dried in a spray-dryer, freeze-dryer, or fluidized bed dryer (*see* **Note 8**). After drying, the microcapsules can be packed in airtight packaging and kept at room temperature until use (*see* **Note 9)**.

3.4 Encapsulation of Probiotics by Spray-Drying

The spray-drying encapsulation technique (Fig. 2) is well established for large-scale industrial applications and is considered an economically viable technique. In this technique, the suspension containing the wall materials and probiotics is atomized in a drying chamber with concurrent hot air, which instantly removes water from the atomized solution [21]. Microcapsules are removed from the drying chamber by a negative pressure cyclone system and can be collected on the drying chamber bottom or in the collection flask (*see* **Note 11**).

1. Preparing the feed solution: Mix 5% (w/v) of WPI in 100 mL of sterile distilled water under stirring at 400 rpm. Then, gently add 1 (w/v) of ALG (*see* **Note 3**), and leave under stirring until the alginate is entirely homogenized (*see* **Note 12**).
2. Addition of cell biomass: Aseptically, an aliquot of the probiotic biomass should be added to the dispersed phase and then

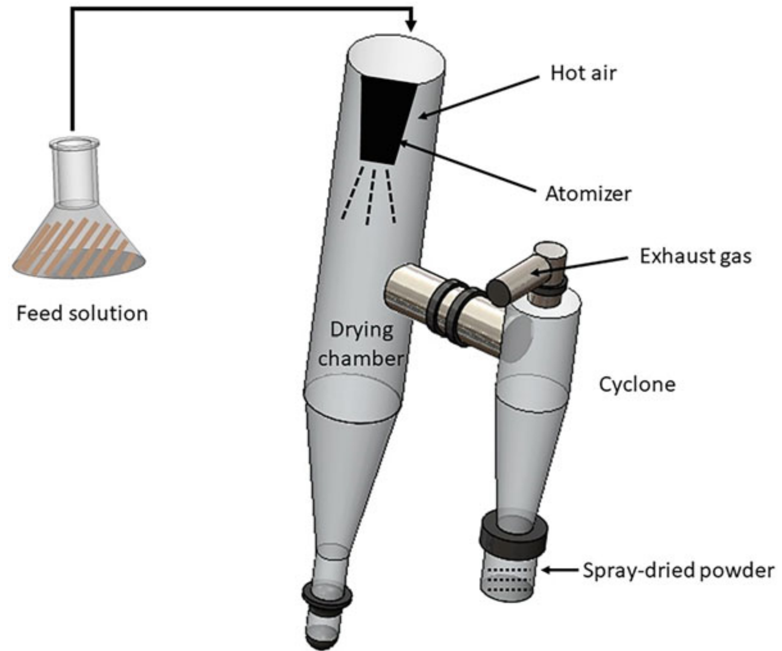

Fig. 2 Spray drying process schematic diagram [22]. (Adapted from [22])

homogenized at 400 rpm for 5 min. It is recommended to add an aliquot with a viable cell count of around 8 to 9 log CFU g^{-1} (*see* **Note 4**).

3. Encapsulation process: Turn on the spray-dryer equipment and operate it with the concurrent flow (*see* **Note 13**) with an inlet temperature of 150 °C and an outlet temperature of 50 °C (*see* **Note 14**). Program the drying airflow of 35 m^3 h^{-1}, and compressor air pressure of 0.7 MPa [23].
4. Then, turn on the peristaltic pump to pump the FS and program supply flow to 20 mL min^{-1}. It was found that slow drying kinetics leads to significant inactivation of the dehydration of *Lactiplantibacillus plantarum*, while a rapid drying rate could instantly stabilize the cells and thereby prevent this inactivation [24]. In addition, a high drying rate during the first stage of drying, when facilitated by hydraulic membrane permeability, may limit bacterial adaptation because of exposure for too short a time to the gradual withdrawal of moisture. It is recommended that the FS be kept under magnetic stirring at room temperature during the encapsulation process (*see* **Note 15**).
5. Before FS entry, sterile distilled water at room temperature must be pumping until stabilization of the inlet temperature.

6. Collecting the dry microcapsules: After complete evaporation of the water, collect the microcapsules from the collector located at the bottom of the equipment, store them in hermetic packages and keep them at room temperature until use (*see* **Note 9**).

The analysis of microcapsules is an important step in the microencapsulation process. Microcapsules must be characterized before use to observe their physical, chemical, and biological properties. Table 2 shows the characteristics of the probiotic microcapsules obtained by the emulsion, extrusion, and spray-drying techniques and the main characterization analyses.

4 Notes

1. You can use other cryoprotectants. MRS for LAB only, if strains from other species (*E. coli*, *Bacillus*, *Saccharomyces*), other broths should be used.
2. Cells in the stationary phase are more resistant and have a higher encapsulation yield than cells in the log phase [33].
3. Alginate should be added gently to not form lumps. You can place it on a foil film and spray it on the solution. Another way to avoid the formation of lumps is to homogenize them in warm water (40–50 °C).
4. Adding an aliquot of *L. rhamnosus* GG with a low viable cell count may compromise delivery to the gut at levels suitable for promoting human health.
5. Any oil can be used. The emulsifying agent is chosen according to the lipophilic hydrophilic balance (LHB); generally, the most used are Tween 80 and Span 80.
6. Using slow agitation rates (400–500 rpm) is recommended. High agitation rates can damage the probiotics'cell wall.
7. Other types of salt can be used for ALG crosslinking, such as calcium citrate. However, it is desirable to use low concentrations. High salt concentrations have a detergent effect, which dissolves bacterial membranes and even causes cell death.
8. Another drying method can be used. However, those are more commonly used. The drying of microcapsules is important both from a microbiological and technological point of view, as it increases the lifespan of microorganisms. In addition, drying the microcapsules makes it possible to incorporate probiotics into low-moisture food matrices.
9. These microcapsules can be used in the products described in other chapters of this edition to improve survivability in processing, storage, and TGI.

Table 2
Characteristics of the probiotic microcapsules obtained by the emulsion, extrusion, and spray-drying techniques and the main characterization analyses

Characteristics of the probiotic microcapsules	How to determine?	Why perform this analysis?	Ideal characteristics	Reference
Size and morphology	**Size:** Laser diffraction method using Mastersizer or dynamic light scattering (DLS) LUMiSizer	The size and morphology of the microcapsules are influenced by the wall materials and the encapsulation technique employed	The microcapsules should not exceed 100 μ in size	[25]
	Morphology: Optical microscopy, fluorescence microscopy, and scanning electron microscopy (SEM)	Microcapsules' size can affect their performance in protection, probiotic delivery, and the sensory quality of the food product incorporated with the microcapsules	They may have a regular shape (e.g., spherical, tubular, and oval) or an irregular shape	
Chemical structure and surface chemistry	Fourier transform infrared spectroscopy (FTIR)	FTIR identifies the functional groups after chemical modification, provides insights into the interaction between microcapsule components, confirms the cross-linking, and probes the degradation of the polymeric matrix	Not applicable	[25]
	Raman spectroscopy	Confirms immobilization and encapsulation of probiotic		
Density: Bulk density and true density	Multi-volume pycnometer and burette containing toluene	The density of microcapsules is a significant factor in the processing, storage, packaging, transportation, and commercialization of the product	Not applicable	[25, 26]

(continued)

Table 2 (continued)

Characteristics of the probiotic microcapsules	How to determine?	Why perform this analysis?	Ideal characteristics	Reference
Porosity	The porosity of the microcapsules can be calculated via the relationship between the bulk and true density of the sample SEM and Brunauer-Emmett-teller (BET) techniques	Porosity is defined as the void fraction in the powder sample. It is an important property that plays an important role in the stability of probiotic powders	Low porosity is desirable, as the presence of pores in the microcapsules can favor the permeation of substances harmful to probiotics, for example, stomach acid	[25, 27]
Water activity (a_w), moisture content, and hygroscopicity	**a_w:** Water activity analyzer (e.g., aqua lab, decagon devices) **Moisture content:** Gravimetrically with heat **Hygroscopicity:** Calculated as the weight of the water absorbed per mass of the sample (%), and can be determined by exposing the microbeads to a NaCl saturated solution for a period of time	Influences the stability of encapsulated probiotics during storage	A high a_w, moisture, and hygroscopicity imply a faster decline in viability during storage a_w around 0.3 is considered satisfactory for dried probiotic microcapsules High hygroscopicity tends to form clusters of microcapsules	[28, 29]
Thermal analysis	Differential scanning calorimetry (DSC)	Thermal stability of wall materials	Not applicable	[30]

Type of order present in powders: Amorphous or crystalline	X-ray diffraction	The crystals could damage the cells, which would reduce the viability of microorganisms, making the amorphous structure interesting Amorphous solids are in general more soluble, and the crystallization may entail a negative impact on the handling properties	Amorphous solids	[31]
Encapsulation process	**Encapsulation yield (EY):** Measured based on the ratio of the number of viable entrapped bacteria to the number of free bacteria	Determines the effectiveness of entrapment within microcapsules and survival of viable cells during the microencapsulation procedure	Microencapsulation can be considered successful when it yields relatively higher EY	[25]
Simulated gastrointestinal conditions	Infogest 2.0	Determines the stability of encapsulated probiotics in simulated gastrointestinal conditions such as mouth, stomach, and intestine	Probiotics should reach the intestine in adequate doses to promote human health	[32]
Thermal stability assay	Bath with controlled temperature	Determines the thermal stability of encapsulated probiotics	Probiotics should be resistant to elevated temperatures Heat-resistant encapsulated probiotics favor the probiotication of thermo-processed foods	[9, 30]
Storage stability	BOD incubator oven	Determines the stability of encapsulated probiotics during storage at different temperatures	It is desirable that probiotics remain stable for long periods	[30]

10. The formation of large particles and the slow production rate are the main disadvantages of this technique for use in the food industry.
11. For probiotic microcapsules, the ideal in bench spray-dryers or pilots is to collect only the product from the collector due to the greater control of the exit temperature of the process.
12. During the process, encapsulated microorganisms can undergo several stresses, including heat stress and dehydration. Encapsulating agents such as gelatin, gum arabic, and cellulose acetate phthalate has been reported as protective agents capable of forming a physical barrier resistant to hot air [21]. In addition, disaccharides are encouraged as they can preserve the structure of probiotic cell proteins and membranes through a connection at sites that previously interacted with water [34].
13. Spray flow can be applied in three ways (concurrent, countercurrent, or mixed flow). However, the choice of spray flow will depend on the direction in which air and liquid (e.g., feed solution) enter the drying chamber. In the first case (concurrent), the product is in contact with the colder air, preferable for drying thermosensitive materials, such as probiotics.
14. The lower the T_{outlet}, the higher the post-drying viability. T_{outlet} is therefore considered to be the principal drying parameter that affects the viability of spray-dried LAB, and any lack of monitoring and control of the latter may be markedly detrimental [21].
15. Agitation prevents materials in solution from settling.

References

1. Camelo-Silva C, Verruck S, Ambrosi A, Di Luccio M (2022) Innovation and trends in probiotic microencapsulation by emulsification techniques. Food Eng Rev 14:462–490. https://doi.org/10.1007/s12393-022-09315-1
2. Hill C, Guarner F, Reid G et al (2014) Expert consensus document: the international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol 11:506–514. https://doi.org/10.1038/nrgastro.2014.66
3. Marcial-Coba MS, Knøchel S, Nielsen DS (2019) Low-moisture food matrices as probiotic carriers. FEMS Microbiol Lett 366:1–11. https://doi.org/10.1093/femsle/fnz006
4. Niño-Vásquez IA, Muñiz-Márquez D, Ascacio-Valdés JA et al (2022) Co-microencapsulation: a promising multi-approach technique for enhancement of functional properties. Bioengineered 13:5168–5189. https://doi.org/10.1080/21655979.2022.2037363
5. de la Cruz P-CA, Ortega D, García-Triana A et al (2020) A brief review of edible coating materials for the microencapsulation of probiotics. Coatings 10:1–36. https://doi.org/10.3390/coatings10030197
6. Corrêa-Filho LC, Moldão-Martins M, Alves VD (2019) Advances in the application of microcapsules as carriers of functional compounds for food products. Appl Sci 9:571. https://doi.org/10.3390/app9030571
7. Bakhtiyari M, Hamidi-Esfahani Z, Barzegar M (2022) Optimization of co-encapsulation of L. plantarum cells and Silybum marianum seed extract and evaluation of protective effect of extract on cells survival in simulated gastrointestinal fluids. LWT 165:113733. https://doi.org/10.1016/J.LWT.2022.113733

8. Rodrigues FJ, Cedran MF, Pereira GA et al (2022) Effective encapsulation of reuterin-producing Limosilactobacillus reuteri in alginate beads prepared with different mucilages/gums. Biotechnol Reports 34:e00737. https://doi.org/10.1016/J.BTRE.2022.E00737
9. Verruck S, de Carvalho MW, de Liz GR et al (2017) Survival of Bifidobacterium BB-12 microencapsulated with full-fat goat's milk and prebiotics when exposed to simulated gastrointestinal conditions and thermal treatments. Small Rumin Res 153:48–56. https://doi.org/10.1016/j.smallrumres.2017.05.008
10. Meireles Mafaldo Í, de Medeiros VPB, da Costa WKA et al (2022) Survival during long-term storage, membrane integrity, and ultrastructural aspects of lactobacillus acidophilus 05 and Lacticaseibacillus casei 01 freeze-dried with freshwater microalgae biomasses. Food Res Int 159:111620. https://doi.org/10.1016/J.FOODRES.2022.111620
11. Thantsha MS, Labuschagne PW, Mamvura CI (2014) Supercritical CO 2 interpolymer complex encapsulation improves heat stability of probiotic bifidobacteria. World J Microbiol Biotechnol 30:479–486. https://doi.org/10.1007/s11274-013-1465-3
12. Hosseini SF, Ansari B, Gharsallaoui A (2022) Polyelectrolytes-stabilized liposomes for efficient encapsulation of lactobacillus rhamnosus and improvement of its survivability under adverse conditions. Food Chem 372:131358. https://doi.org/10.1016/j.foodchem.2021.131358
13. Xu C, Ma J, Wang W et al (2022) Preparation of pectin-based nanofibers encapsulating lactobacillus rhamnosus 1.0320 by electrospinning. Food Hydrocoll 124:107216. https://doi.org/10.1016/J.FOODHYD.2021.107216
14. Martinez CJ, Kim JW, Ye C et al (2012) A microfluidic approach to encapsulate living cells in uniform alginate hydrogel microparticles. Macromol Biosci 12:946–951. https://doi.org/10.1002/mabi.201100351
15. Yao M, Lu Y, Zhang T et al (2021) Improved functionality of Ligilactobacillus salivarius Li01 in alleviating colonic inflammation by layer-by-layer microencapsulation. Biofilmes e Microbiomas 58:1–10. https://doi.org/10.1038/s41522-021-00228-1
16. Mirzamani SS, Bassiri AR, Tavakolipour H et al (2021) Survival of fluidized bed encapsulated lactobacillus acidophilus under simulated gastro-intestinal conditions and heat treatment during bread baking. J Food Meas Charact 15:5477–5484. https://doi.org/10.1007/s11694-021-01108-0
17. Kuo CC, Clark S, Qin H, Shi X (2022) Development of a shelf-stable, gel-based delivery system for probiotics by encapsulation, 3D printing, and freeze-drying. LWT 157:113075. https://doi.org/10.1016/J.LWT.2022.113075
18. Camelo-Silva C, da Barros EL, Verruck S et al (2022) How ice cream manufactured with concentrated milk serves as a protective probiotic carrier? An in vitro gastrointestinal assay. Food Sci Technol 42:e28621. https://doi.org/10.1590/fst.28621
19. Martín MJ, Lara-Villoslada F, Ruiz MA, Morales ME (2015) Microencapsulation of bacteria: a review of different technologies and their impact on the probiotic effects. Innov Food Sci Emerg Technol 27:15–25. https://doi.org/10.1016/j.ifset.2014.09.010
20. Sultana M, Chan ES, Pushpamalar J, Choo WS (2022) Advances in extrusion-dripping encapsulation of probiotics and omega-3 rich oils. Trends Food Sci Technol 123:69–86. https://doi.org/10.1016/J.TIFS.2022.03.006
21. Huang S, Vignolles ML, Chen XD et al (2017) Spray drying of probiotics and other food-grade bacteria: a review. Trends Food Sci Technol 63:1–17. https://doi.org/10.1016/j.tifs.2017.02.007
22. de Liz GR, Verruck S, Canella MHM et al (2020) Stability of bifidobacteria entrapped in goat's whey freeze concentrate and inulin as wall materials and powder properties. Food Res Int 127:108752. https://doi.org/10.1016/j.foodres.2019.108752
23. Verruck S, Santana F, de Olivera MC, Prudencio ES (2018) Thermal and water sorption properties of Bifidobacterium BB-12 microcapsules obtained from goat's milk and prebiotics. LWT – Food Sci Technol 98:314–321. https://doi.org/10.1016/J.LWT.2018.08.060
24. Perdana J, Bereschenko L, Fox MB et al (2013) Dehydration and thermal inactivation of lactobacillus plantarum WCFS1: comparing single droplet drying to spray and freeze drying. Food Res Int 54:1351–1359. https://doi.org/10.1016/j.foodres.2013.09.043
25. Razavi S, Janfaza S, Tasnim N et al (2021) Microencapsulating polymers for probiotics delivery systems: preparation, characterization, and applications. Food Hydrocoll 120:106882. https://doi.org/10.1016/J.FOODHYD.2021.106882
26. Arepally D, Goswami TK (2019) Effect of inlet air temperature and gum Arabic concentration on encapsulation of probiotics by spray drying. LWT 99:583–593. https://doi.org/10.1016/j.lwt.2018.10.022

27. Bhusari SN, Muzaffar K, Kumar P (2014) Effect of carrier agents on physical and microstructural properties of spray dried tamarind pulp powder. Powder Technol 266:354–364. https://doi.org/10.1016/J.POWTEC.2014.06.038
28. de Paula DA, EMF M, de Costa NA et al (2019) Use of gelatin and gum arabic for microencapsulation of probiotic cells from lactobacillus plantarum by a dual process combining double emulsification followed by complex coacervation. Int J Biol Macromol 133:722–731. https://doi.org/10.1016/j.ijbiomac.2019.04.110
29. Silva MP, Tulini FL, Martins E et al (2018) Comparison of extrusion and co-extrusion encapsulation techniques to protect lactobacillus acidophilus LA3 in simulated gastrointestinal fluids. LWT – Food Sci Technol 89:392–399. https://doi.org/10.1016/j.lwt.2017.11.008
30. Beldarrain-Iznaga T, Villalobos-Carvajal R, Leiva-Vega J, Sevillano Armesto E (2020) Influence of multilayer mi;croencapsulation on the viability of lactobacillus casei using a combined double emulsion and ionic gelation approach. Food Bioprod Process 124:57–71. https://doi.org/10.1016/j.fbp.2020.08.009
31. Dantas A, Verruck S, Machado Canella MH et al (2021) Current knowledge about physical properties of innovative probiotic spray-dried powders produced with lactose-free milk and prebiotics. LWT 151:112175. https://doi.org/10.1016/J.LWT.2021.112175
32. Brodkorb A, Egger L, Alminger M et al (2019) INFOGEST static in vitro simulation of gastrointestinal food digestion. Nat Protoc 14:991–1014. https://doi.org/10.1038/s41596-018-0119-1
33. Jananee J, Preeti S (2017) Molecular basis of stationary phase survival and applications. Front Microbiol 8:2000. https://doi.org/10.3389/fmicb.2017.02000
34. Perdana J, Fox MB, Siwei C et al (2014) Interactions between formulation and spray drying conditions related to survival of lactobacillus plantarum WCFS1. Food Res Int 56:9–17. https://doi.org/10.1016/j.foodres.2013.12.007

Chapter 15

Paraprobiotics Preparation for Use in Food and Beverages

Cássia Pereira Barros, Roberto Pessanha da Silva Pires, and Jonas de Toledo Guimarães

Abstract

In recent years, the high demand for functional products has driven the food industry to produce healthier foods. In this context, the incorporation of probiotics has been an excellent strategy to improve the nutritional value of food products. The current definition of probiotics indicates that microorganisms need to be alive to provide health benefits to the consumer. However, scientific evidence has indicated that bacterial viability is not necessary for health promotion. Paraprobiotics are inactivated microbial cells or cell fractions that, when ingested, confer health benefits. Additionally, they are safer, more economical, and stable therapeutic options for industrial processes and commercialization. Therefore, this chapter provides detailed protocols for obtaining potential paraprobiotics for use in food and beverages.

Key words Functional foods, Nonviable microorganisms, Inactivated probiotics, Postbiotics, Inactivation methods

1 Introduction

Probiotics play an important role in promoting health and well-being through the prophylaxis and therapeutic intervention of various disorders or diseases. Although the primary function of probiotic bacteria is the restoration of healthy intestinal microbiota and, consequently, improvement of gastrointestinal disorders [1], beneficial systemic (extra-intestinal) effects via mediators of the immune, endocrine, and nervous systems have been increasingly explored [2–4].

According to the International Scientific Association of Probiotics and Prebiotics (ISAPP), probiotics are "live microorganisms that when administered in adequate amounts, confer a health benefit on the host" [5]. Therefore, according to this definition, bacterial viability is essential for the beneficial effects associated

The original version of this chapter was revised. The correction to this chapter is available at https://doi.org/10.1007/978-1-0716-3187-4_18

Adriano Gomes da Cruz et al. (eds.), *Probiotic Foods and Beverages: Technologies and Protocols*, Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-3187-4_15,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2023, Corrected Publication 2023

with its consumption to be observed. However, certain aspects inherent to the use of live bacteria must be considered, such as restricted application in certain food matrices considered stressful substrates; high costs with management and control of temperature, oxygen, and humidity to maintain probiotic viability during processing, storage, and commercialization [6, 7]; and greater risks of causing adverse effects in individuals with weakened immune systems (elderly, transplanted, newly premature babies, etc.) [7–9].

On the other hand, in recent decades, several scientific studies have shown that dead or inactivated probiotics are also capable of promoting biological activity in the host through modulation of the innate and adaptive immune system, in addition to exerting anti-inflammatory, anti-proliferative, antioxidant, and antagonistic effects against pathogens [10, 11]. The term "paraprobiotics" was created to name inactivated microbial cells or cell fractions that, when ingested, confer health benefits to the consumer [12]. However, in 2021, ISAPP proposed to update the concept of postbiotics to "preparation of inanimate microorganisms and/or their components that confer a benefit to the health of the host" [13]. Thus, the concept initially used to refer to the metabolites produced by microorganisms would also include nonviable microbial cells. However, paraprobiotics continue to be widely used by the scientific community.

The addition of paraprobiotics, as bioactive ingredients, represents a potential opportunity for food and pharmaceutical industries to diversify the niche of functional products, nutraceuticals, and alternative medicines. When comparing the use of inactivated probiotic bacteria to their viable counterparts, the following advantages can be highlighted: they have no or minimal interaction with the components of the food matrix, which makes it possible to include them in any food without altering the sensory characteristics and, consequently, extends the commercial validity of the product; there are no restrictions on their consumption as a food supplement and they are stable at room temperature, providing convenience for large-scale production and significant savings for manufacturers as they do not require a cold chain during storage and distribution [7, 10].

In recent years, many formulations supplemented with paraprobiotics in dairy matrices (fermented milks and dairy drinks) and non-dairy matrices (isotonic drinks and pasta) have been successfully developed, resulting in several benefits (improvement of intestinal function and microbiota, reduction of chronic stress and improvement in sleep quality, anxiety relief, postprandial glycemic control, reduction of glucose and total cholesterol levels, among others) [14–18]. Therefore, the objective of this chapter is to provide, in detail, protocols for the preparation of potential paraprobiotics with the aim of conferring functionality to foods and beverages.

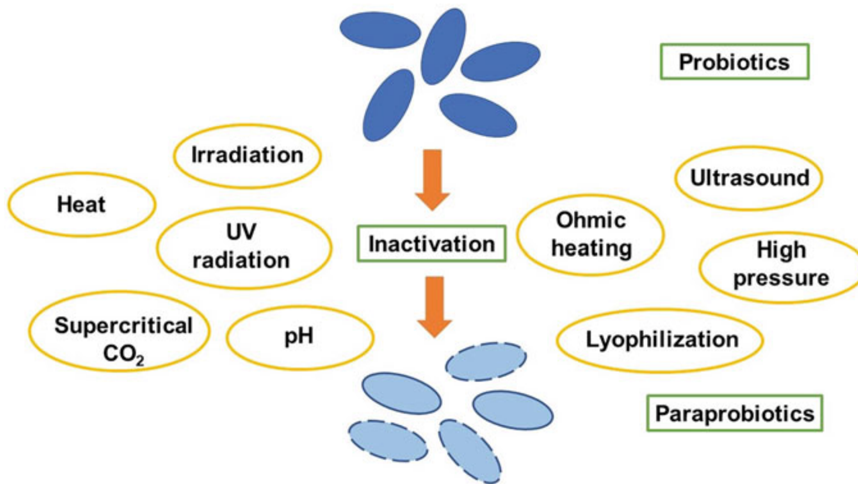

Fig. 1 Examples of inactivation processes applied to generate paraprobiotics

1.1 How to Produce Paraprobiotics?

The production of paraprobiotics consists of the inactivation of probiotic microorganisms either by traditional or emerging methodologies, as examples (Fig. 1): application of heat (pasteurization, sterilization, or tindalization), pH changes (low and high), gamma irradiation, ultrasound, supercritical CO_2 [19], lyophilization [20], ultraviolet radiation [21], high pressure [22], ohmic heating [23], and chemical agents. In general, such processes can damage the cell membrane, break genomic DNA, coagulate cytoplasmic proteins and/or compromise different physiological functions (inactivation of key enzymes or deactivation of membrane selectivity) [19], which renders probiotic bacteria unable to grow in culture media.

It is important to emphasize that each inactivation process has its own mechanisms of action and, therefore, will affect the bacterial structural and physiological components in different ways and, thus, influence the paraprobiotic immunomodulatory activity [10, 12]. In this sense, it is essential that the methods and operational conditions chosen, in addition to inactivating, are able to preserve the metabolic activity and the integrity of the cell membrane, in order to maintain the functionality initially provided by live probiotics [12, 18, 19].

2 Materials

1. Probiotic strain(s) can be acquired in lyophilized form for direct addition (Direct Vat Set – DVS) or can be isolated from fermented products and functional food (as starter cultures for sausages, dairy products, etc.), as well as from the intestine of humans and healthy animals. We particularly prefer to use Direct Vat Set (DVS) cultures for practicality.

2. Laboratory tools – Petri dishes (90 mm diameter), spatula, glass pipet, micro-pipet, laboratory glassware, sterile tubes, sterile plastic bag and sterile loop;
3. Laboratory equipment – analytical balance, microwave or stove, autoclave, bacteriological incubator, centrifuge, spectrophotometer, vortex mixer, thermostatic bath, and sample homogenizer;
4. Distilled water;
5. Culture media – MRS (De Man Rogosa and Sharpe) broth and MRS agar. Culture media should be prepared according to the manufacturer's instructions provided on the label (*see* **Note 1**). After accurately weighing the dehydrated medium, pour all ingredients in an Erlenmeyer flask or any volumetric flask and dilute to 1 L with distilled water. Then mix until complete dissolution using a glass rod and heat (*see* **Notes 2** and **3**). Afterwards, verify that the pH of the media is adequate and proceed to sterilization in an autoclave at 121 °C for 15 min. After removal from the autoclave, identify them correctly with name and date.
6. Phosphate-buffered saline (PBS, pH 7), (*see* **Note 4**);
7. Chemical reagent – 30% hydrogen peroxide (H_2O_2);

3 Methods

3.1 Probiotics Strains and Cell Suspensions Preparation

For the isolation of probiotic strains from fermented foods or dairy products, the following procedures must be performed:

1. Weigh 25 g of the sample and transfer it quantitatively to a sterile plastic bag, add 250 mL of phosphate-buffered saline (PBS, pH 7) and place in the sample homogenizer for 1 min at 300 rpm (*see* **Note 5**).
2. Make tenfold dilutions by adding the 1 mL aliquot of the sample to 9 mL of phosphate-buffered saline (PBS, pH 7), homogenize each dilution using a vortex mixer. Transfer 1 mL aliquot of each selected dilution to Petri dishes and add 12–15 mL of MRS agar (pour plate technique), as illustrated in Fig. 2 (*see* **Note 6**). Subsequently, incubate the plates under anaerobic conditions at 37 °C for 24–48 h (*see* **Note 7**).
3. Select colonies with a circular shape, creamy texture, and white color to be cultivated in MRS broth for 24 h under anaerobic conditions at 37 °C (Fig. 3). After the incubation period, seed again on plates containing MRS agar until complete isolation is achieved.

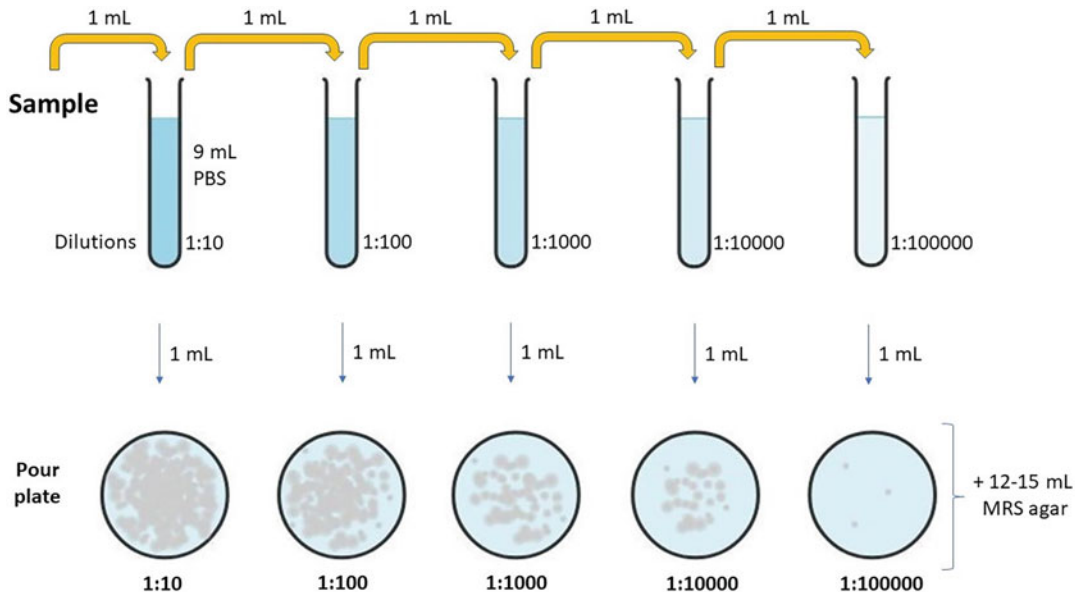

Fig. 2 Plate counting method by the pour plate technique. (Source: The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license (https:/creativecommons.org/licenses/by/3.0/) and using Chemix (https:/chemix.org))

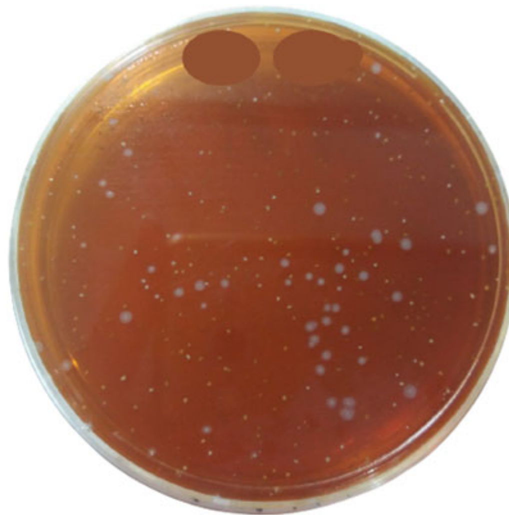

Fig. 3 Example of color, texture, and shape of colonies that must be selected for new cultivation until complete purification is achieved

4. The isolated colonies must be analyzed in relation to their morphological characteristics by the Gram stain method. Gram-positive colonies should proceed for the Catalase test.

   Catalase test.

   Procedure – using a sterile loop, add colony to a glass slide and add 2 drops of 30% (v/v) hydrogen peroxide (H_2O_2).

   Data interpretation: the formation of gas bubbles indicates the production of catalase enzyme by the test bacterium, while no bubble formation is negative for catalase.

5. Purified Gram positive and catalase negative strains are considered presumptive lactic acid bacteria. They can be kept as frozen stocks in 20% (v/v) glycerol at −20 °C until time of use.
6. Provide genotypic identification of the isolated strain for deposit.

On the other hand, if you have obtained Direct Vat Set (DVS) pouches of commercial lyophilized probiotic culture, simply activate them as follows:

1. Activate probiotic cultures in MRS broth for 24–48 h at 37 °C under anaerobic conditions.
2. Harvest cells by centrifugation at 2700 x g, 4 °C for 10 min, wash twice with phosphate-buffered saline (PBS, pH 7) and resuspend in the same buffer solution [19].
3. Adjust the cell concentration of bacterial suspensions with phosphate-buffered saline (PBS, pH 7) to achieve an optical density at 600 nm (OD 600 nm) of 0.7 to 0.8 (corresponding to a cell concentration of around 7–8 log CFU/ml) (*see* **Note 8**).

3.2 Inactivation Treatment for Paraprobiotic Production

As previously mentioned, there are several inactivation processes that can be used to obtain potential paraprobiotics (item 2). The application of heat has been the most used methodology due to its practicality and low cost. The mechanisms of thermal inactivation may involve damage to cell membrane, loss of nutrients and ions, ribosome aggregation, rupture of DNA filaments, essential enzyme inactivation, and protein coagulation. In general, heat treatments of bacterial suspensions use a temperature range between 70 and 100 °C (*see* **Note 9**). In addition, inactivation can also be achieved through the interspersed use of elevated temperatures followed by incubation periods with milder temperatures that can be ambient, cooling, or freezing [24]. Preliminary tests are recommended to determine the operational conditions (time x temperature binomial) that will result in the inactivation of probiotic cell suspensions (*see* **Note 10**).

Procedures:

1. Adjust the thermostatic bath according to the desired temperature for the treatment.
2. After reaching the target temperature, immerse the tubes containing the cell suspensions (*see* **Note 11**).
3. At the end of the treatment, the bacterial suspensions must be immediately cooled in an ice bath at 4 °C in order to stop heating, and then evaluate them regarding the effectiveness of the inactivation treatments under the conditions considered by the traditional method of plate count.

3.3 Assessment of Probiotic Cell Viability After Inactivation Treatment

All bacterial suspensions, treated and untreated (control), should be analyzed for the presence or absence of CFU growth. For the production of potential paraprobiotics, the absence of CFU in plates with pure cell suspensions is necessary, thus indicating that the treatment and the respective inactivation condition were, in fact, effective for obtaining paraprobiotics (*see* **Note 12**). Otherwise, the presence of CFU indicates that the inactivation of the probiotic cells was not achieved in the operational parameters tested.

Procedures:

1. Make serial dilutions of treated and untreated cell suspensions in sterile tubes containing phosphate-buffered saline (PBS, pH 7) in a 1:10 ratio.
2. Inoculate the dilutions in MRS agar using the pour plate technique, as described in item 2 of topic 3.1.
3. Incubate the plates under anaerobic conditions for a period of 72 h, considering the possibility of growth retardation.
4. After the incubation period, the plates must be analyzed in order to verify the growth or not of CFU in the tested inactivation conditions.
5. If there is growth of colonies (live probiotics), they must be analyzed for their morphological characteristics by the GRAM staining method and catalase test.

4 Notes

1. In order to avoid waste, it is recommended to prepare just enough medium for each test. After cooling the MRS agar, distribute approximately 15 mL of the sterile medium in a Petri dish. Then just store the Petri dishes below 5 °C until further use.
2. Be extremely careful when heating to prevent the culture medium to be overheated or burnt.

3. During the preparation of MRS agar, use a glass rod to place a drop of the medium on a cold surface. If after a few seconds it solidifies, it indicates that it is ready to be sterilized in the autoclave.
4. Phosphate-buffered saline (PBS, pH 7) can be replaced with other saline solutions such as (0.85% NaCl, pH 7) sterile saline or even 0.1% (w/v, pH 7) peptone water.
5. All bacteriological procedures need to be performed using a Bunsen burner in a clean and closed working area or inside a sterile laminar flow hood or similar.
6. Before adding MRS agar, make sure it is not too hot. After pouring on the plate, homogenize slowly so as not to spread it on the wall or cover of the plate. Wait for the medium to solidify completely before taking it to a bacteriological incubator.
7. An incubation period of 24 h is required for the growth of microorganisms of the *Lactobacillaceae* family and 48 h for microorganisms belonging to the genus *Bifidobacterium* spp. [23].
8. If the laboratory does not have a spectrophotometer available, the adjustment of cell density of probiotic suspensions can be performed using the McFarland nephelometric scale.
9. Typically, paraprobiotic microorganisms cultivated on an industrial scale through heat treatments have been able to retain their main functionalities from viable microbes (probiotics) at the intestinal level [24].
10. Although so far few studies have evaluated the impact of different inactivation treatments on bacterial structure and components [19, 22, 23, 25], there are advanced techniques that can be complementary to the plate counting method and help in the choice of methods/more suitable inactivation conditions in order to maintain the probiotic properties. Flow cytometry consists of an automated instrument that, associated with fluorescent dyes, allows the simultaneous evaluation of several cellular parameters, such as alterations in structural and metabolic properties. In this way, it is possible to assess the extent of cell damage induced by inactivation processes, generating accurate results in real time [26].
11. During heat treatment in the thermostatic bath, it is recommended to use a thermocouple and multimeter to adjust and control the temperature more precisely.
12. It is important to highlight that the probiotic inactivation process produces potential paraprobiotics. However, in vitro, preclinical or clinical tests are extremely necessary to confirm whether, in fact, the permanence of the functionality initially provided by probiotic microorganisms.

References

1. Sanders ME, Merenstein DJ, Reid G, Gibson GR, Rasrall RA (2019) Probiotics and prebiotic0s in intestinal health and disease: from biology to the clinic. Nat Rev Gastroenterol Hepatol 16:605–616
2. Singh K, Rao A (2021) Probiotics: a potential immunomodulator in COVID-19 infection management. Nutr Res 87:1–12
3. Paul P, Kaul R, Harfouche M et al (2022) The effect of microbiome-modulating probiotics, prebiotics and synbiotics on glucose homeostasis in type 2 diabetes: a systematic review, meta-analysis, and meta-regression of clinical trials. Pharmacol Res 185:106520
4. Zhu H, Tian P, Zhao J, Zhang H, Wang G, Chen W (2022) A psychobiotic approach to the treatment of depression: a systematic review and meta-analysis. J Funct Foods 91: 104999
5. Hill C, Guarner F, Reid G et al (2014) Expert consensus document. The international scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol 11:506–514
6. Terpou A, Papadaki A, Lappa IK et al (2019) Probiotics in food systems: significance and emerging strategies towards improved viability and delivery of enhanced beneficial value. Nutrients 11:1591
7. Barros CP, Guimarães JT, Esmerino EA et al (2020) Paraprobiotics and postbiotics: concepts and potential applications in dairy products. Curr Opin Food Sci 32:1–8
8. Deshpande G, Athalye-Jape G, Patole S (2019) Para-probiotics for preterm neonates – the next frontier. Nutrients 10:871–879
9. Zawistowska-Rojek A, Tyski S (2018) Are probiotic really safe for humans? Pol J Microbiol 67:251–258
10. De Almada CN, Almada CN, Martinez RCR et al (2016) Paraprobiotics: evidences on their ability to modify biological responses, inactivation methods and perspectives on their application in foods. Trends Food Sci Technol 58:96–114
11. Siciliano RA, Reale A, Mazzeo MF et al (2021) Paraprobiotics: a new perspective for functional foods and nutraceuticals. Nutrients 13:1225
12. Taverniti V, Guglielmetti S (2011) The immunomodulatory properties of probiotic microorganisms beyond their viability (ghost probiotics: proposal of paraprobiotic concept). Genes Nutr 6:261–274
13. Salminen S, Collado MC, Endo A et al (2021) The international scientific Association of Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of postbiotics. Nat Rev Gastroerol Hepatol 18: 649–667
14. Sawada D, Sugawara T, Ishida Y et al (2016) Effect of continuous ingestion of a beverage prepared with *lactobacillus gasseri* CP2305 inactivated by heat treatment on the regulation of intestinal function. Food Res Int 79:33–39
15. Nishida K, Sawada D, Kuwano Y et al (2017) Daily administration of paraprobiotic lactobacillus gasseri CP2305 ameliorates chronic stress-associated symptoms in Japanese medical students. J Funct Foods 36:112–121
16. Sawada D, Kuwano Y, Tanaka H et al (2019) Daily intake of lactobacillus gasseri CP2305 relieves fatigue and stress-related symptoms in male university Ekiden runners: a double-blind, randomized, and placebo-controlled clinical trial. J Funct Foods 57:465–476
17. Barros CP, Grom LC, Guimarães JT et al (2021) Paraprobiotic obtained by ohmic heating added in whey-grape juice drink is effective to control postprandial glycemia in healthy adults. Food Res Int 140:109905
18. Almada CN, Almada-Érix CN, Costa WKA et al (2021) Wheat-durum pasta added of inactivated *Bifidobacterium animalis* decreases glucose and total cholesterol levels and modulates gut microbiota in healthy rats. Int J Food Sci Nutr. https://doi.org/10.1080/09637486.2021.1877261
19. Almada CN, Almada-Érix CN, Bonatto MS et al (2021) Obtaining paraprobiotics from *Lactobacilus acidophilus*, *Lacticaseibacillus casei* and *Bifidobacterium animalis* using six inactivation methods: impacts on the cultivability, integrity, physiology, and morphology. J Funct Foods 87:104826
20. Nakamura S, Mitsunaga F (2018) Anti-allergic effect of para-probiotics from nonviable acetic acid bacteria in ovalbumin-sensitized mice. Food Nutr Sci 9:1376–1385
21. Gayán E, Alvarez I, Condón S (2013) Inactivation of bacterial spores by UV-C light. Innov Food Sci Emerg Technol 19:140–145
22. Ananta E, Knorr D (2009) Comparison of inactivation pathways of thermal or high pressure inactivated *lactobacillus rhamnosus* ATCC 53103 by flow cytometry analysis. Food Microbiol 26:542–546

23. Barros CP, Pires RPS, Guimarães JT et al (2021) Ohmic heating as a method of obtaining paraprobiotics: impacts on cell structure and viability by flow cytometry. Food Res Int 140:110061
24. Pique N, Berlanga M, Miñna-Galbis D (2019) Health benefits of heat-killed (Tyndallized) probiotics: an overview. Int J Mol Sci 20:2534
25. Pires RPS, Guimarães JT, Barros CP et al (2021) Ohmic heating increases inactivation and morphological changes of salmonella sp. and the formation of bioactive compounds in infant formula. Food Microbiol 97:103737
26. Wilkinson MG (2018) Flow cytometry as a potential method of measuring bacterial viability in probiotic products: a review. Trends Food Sci Technol 78:1–10

Chapter 16

Postbiotics Preparation for Use in Food and Beverages

Jonas de Toledo Guimarães, Cássia Barros, Houshmand Sharafi, Mehran Moradi, Erick A. Esmerino, and Adriano Gomes da Cruz

Abstract

Postbiotics are considered novel bioactive compounds, since there is not yet a consensus about its definition; however, many postbiotic compounds are already used in the food industry to provide food preservation, in the manufacture of bioactive food packaging, control bacterial biofilm, or provide functional effects to food products. The postbiotics can be applied to food as a mixture (several compounds, often called cell-free supernatant) or a separated form; however, there are issues about the production of these postbiotics and the practical application to different food products. This chapter aims to present the most used methodologies and the details about its production and application.

Key words Bioactive compounds, Postbiotic metabolites, Probiotics, Cell-free supernatant, Food preservation

1 Introduction

Probiotics and prebiotics are well-known and defined functional compounds, but other components derived from microorganisms, which also provide health benefits but refer only to inanimate microorganisms and/or their components such as paraprobiotics, ghostbiotics, heat-inactivated probiotics, and postbiotics, still cause divergences in the scientific community [1]. The most recent definition of postbiotics from the International Scientific Association for Probiotics and Prebiotics (ISAPP), states that postbiotics is "a preparation of inanimate microorganisms and/or their components that confers a health benefit on the host," therefore, a postbiotic to be considered effective must contain inactivated microbial cells or cell components, with or without metabolites, and promote health effects [2]. However, the above definition also includes inactivated microorganisms that is commonly known as paraprobiotics, therefore, other authors still consider the original definition of postbiotics presented by Tsilingiri, Rescigno [3] as "any factor

Adriano Gomes da Cruz et al. (eds.), *Probiotic Foods and Beverages: Technologies and Protocols*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-3187-4_16,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2023

resulting from the metabolic activity of a probiotic or any released molecule capable of conferring beneficial effects to the host in a direct or indirect way".

In this context, due to the lack of a consensus regarding postbiotics, many different bioactive compounds can be considered postbiotics, since they did not fit the traditional definitions of probiotics, prebiotics, and paraprobiotics. According to Moradi et al. [4], the term postbiotics can be used to characterize the "bioactive soluble factors (products or metabolic byproducts) produced by food-grade microorganisms during the growth and fermentation in complex microbiological culture (in this case named cell-free supernatant [CFS]), food, or gut, which exert some benefits to the food or host." Furthermore, according to [5], "a postbiotic must be derived from a well-defined microorganism or combination of microorganisms for which genomic sequences are known and prepared using a delineated technological process of biomass production and inactivation, which can be reliably reproduced." Currently, several postbiotic molecules are being identified by researchers as a result of extracellular metabolites production or release of intracellular components from microbial cells. These components include secreted proteins/peptides, bacteriocins such as acidophilin, reuterin, and bifidin, cell-free supernatant, organic acids (e.g. lactic acid and acetic acid), vitamins, short-chain fatty acids (e.g. butyric acid and propionate), and neurotransmitters, biosurfactants. [6].

Probiotics are recognized for their diverse effects on consumer health as long as the supplementation is long enough to colonize gut microbiota and then produce their beneficial compounds. Therefore, the bioactive compounds produced by probiotics are the main factors responsible for their effects, and these compounds, named postbiotics, can most of the time provide effects similar to the living microorganisms. The advantage of using postbiotics in food and beverages instead of probiotics is the absence or reduction of changes in product's characteristics, such as physicochemical and sensory [4]. Despite the known effects of probiotics and their metabolites, other non-probiotic microorganisms may also produce metabolites with significant effects on the host or food product, being, therefore, considered a postbiotic.

In order to use postbiotics in food and beverages, they must be properly prepared to maintain their activity, remove the living microorganisms and reduce the undesired effects (e.g., sensory changes) on food products. Postbiotics prepared from probiotic or other known microorganisms, mostly lactic acid bacteria can present different bioactive compounds such as organic acids, short-chain fatty acids, carbohydrates, antimicrobial peptides, enzymes, vitamins, cofactors, immune-signaling compounds, and complex agents [4]. These postbiotics can be applied with different

objectives, such as food preservation, in the manufacture of bioactive food packaging, controlling bacterial biofilm, or providing functional effects to food products [4, 6, 7].

The postbiotic can be applied to food in a mixture (several compounds, often called cell-free supernatant) or in a separated form (preparation derivative), which will depend on the objective of the application or resources available for postbiotic preparation since the preparation of a specific postbiotic is more expensive and complex than the preparation of a mixture [4]. Therefore, the objective of this chapter is to present the main methodologies used for postbiotics preparation in order to use it in food and beverages.

2 Materials

2.1 Consumables

- Ammonium sulfate
- Ascorbic acid
- Acetic acid
- Catalase
- Dialysis tubing (1 kDa cut-off)
- Erlenmeyers (100, 250 and 1000 mL)
- Glass tubes (10 mL)
- Glass bottles (100 and 1000 mL)
- L-cysteine
- Lithium chloride
- Meta-phosphoric acid
- MRS (De Man, Rogosa and Sharpe) broth (for lactic acid bacteria)
- NaCl solution 0.9% (v/v)
- NaOH
- Polyethylene bags
- Potassium cyanide solution
- Sterile distilled water and buffer solution (phosphate buffer saline; PBS, 0.01 M phosphate, pH 7.2)
- Syringe or Buchner filter (0.22 and 0.45-μm pore size)
- Target microorganism (s) in lyophilized form

2.2 Equipment

- Automated pipette gun and Micropipette (100–1000 μL and 1000–10,000 μL)
- Autoclave
- Bunsen burner

- Freeze dryer
- Lab vortex mixer
- Laboratory analytical balance
- Laminar flow cabinet
- Magnetic stirrer
- pH meter
- Rotary evaporator
- Refrigerated centrifuge
- Refrigerator (4, −20 and −70 °C)
- Spray dryer
- Standard incubator and CO_2 incubator
- UV spectrometer
- Vacuum filtration glassware system
- Vacuum pump
- Water bath

3 Methods

3.1 Cell-Free Supernatant (CFS)

This form of postbiotic preparation may contain products from microbial metabolisms such as metabolites synthesized by the microorganism on culture/food ingredients or structural substances produced by them. It is important to highlight that the microbial products are mainly related to the type of bacterial strain and culturing medium, which contain only soluble factors such as products or metabolic by-products that have been secreted into the medium during bacterial growth [7].

After microbial propagation to produce CFS, the bacterial cells may be inactivated/ disrupted (e.g., enzymatic treatment, thermal treatment, sonication, high pressure, Ultraviolet rays, ohmic technology) in order to release new substances into the postbiotic mixture such as intracellular metabolites and cell wall-derived materials. Treatment parameters in every process may vary according to the type of microorganism and the target postbiotics. Non-thermal processes were less reported and heat treatment is still the method of choice in most cases [8]. Then, the postbiotic mixture, independently of the post-propagation treatment, is centrifuged and/or filtered to separate bacterial cells from postbiotic metabolites [9].

Most of the postbiotic CFS preparations are carried out according to Fig. 1, however, some modifications may be applied to the fermentation medium and the microbial strain to obtain different postbiotics.

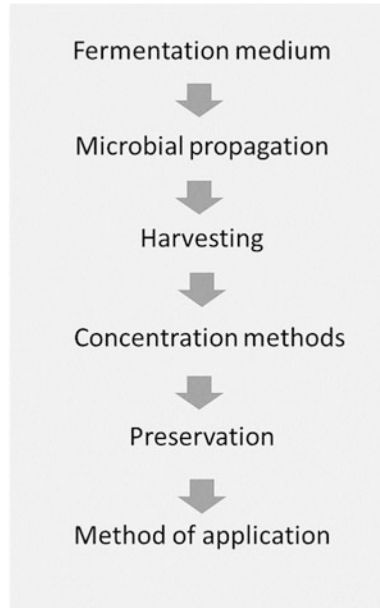

Fig. 1 General flowchart of a postbiotic cell-free supernatant preparation

3.1.1 Fermentation Medium

Most postbiotic preparations are made with lactic acid bacteria (LAB); therefore, the MRS broth is commonly used [7]; however, other media can be used, including some based on dairy ingredients such as modified cheese whey and milk permeate [10]. Despite the greater importance of LAB on postbiotics production, other microorganisms may also be used such as fungal species [11], but the fermentation medium will change for fungal species, since their metabolism are different than LAB.

In case of preservation of the microbial strains, they can be kept frozen (−80 °C). However, before freezing, the concentrated/centrifuged probiotic cells must be added of 20% glycerol [12] to maintain cell integrity during freezing and thawing [13]. Another microbial preservation method is lyophilization, in which the product can be kept in refrigeration or even at room temperature after the process. In addition, many microorganisms and protective cultures of certain reputable companies are commercially available in frozen and freeze-dried concentrated cultures (known as direct vat set (DVS) or direct-to-vat inoculation (DVI) cultures), and can simply be used according to provider's instruction.

The culture characteristics, medium composition, incubation conditions, and cultivation time can affect metabolites production by LAB. It was reported that postbiotics from different LAB strains prepared with low-heat milk revealed significantly higher antifungal activity than those prepared with milk permeate [10]. The cultivation of probiotics in MRS media supplemented with glucose, yeast extract, surfactants, or emulsifiers could increase the production of postbiotic products such as bacteriocin-inhibitory activity, proteinaceous and therapeutic substance with antimicrobial activity [7].

Evidences tend to support the hypothesis that a mixture of multi-strains is more effective than a mono-strain to produce postbiotics. Furthermore, coculturing can facilitate the expression of ribosomal synthesized antimicrobial agents (AAs) like bacteriocins in LAB, also leading to the discovery of novel AAs [7], but in some cases, a combination of two or three strains does not work [14], requiring a compatibility test before the experiment. It has also been shown in laboratory that postbiotics can be produced by fermentation with proteolytic starter cultures, and if the pH is maintained close to neutrality during fermentation, it may improve the release of postbiotic compounds [15].

Procedure

For probiotic bacteria strain cultivation to produce postbiotic compounds some important steps must be carefully performed for safe production, as briefly presented in Fig. 2. All material and equipment used during the procedure must be sterile or autoclaved (121 °C for 15 min). The work must be done in aseptic conditions, preferably using a laminar flow cabinet.

1. *Culture media preparation*: Currently, the most used probiotic strains are from *Lactobacilli* and *Bifidobacteria* families, being the MRS media the best option to grow them [9, 10, 16–18] (*see* **Note 1**).
2. *Pre-activation*: If a microbial strain is lyophilized or kept frozen, a pre-activation is required (*see* **Note 2**), incubating the microorganisms usually in glass tubes containing MRS broth (10 mL) at 32–37 °C for a specific period of time (mostly 48 h) under aerobic or anaerobic conditions depending on the type of strain. Some strains require special nutrients and inhibitors (*see* **Note 3**).
3. *Inoculum preparation*: The microbial suspension can be centrifuged at 5000 g for 15 min and the obtained pellet is washed using 0.9% (v/v) NaCl solution or PBS three times. Finally, the obtained bacterial cells are suspended in PBS to standardize the microbial concentration before inoculation and propagation (*see* **Note 4**) [19].

3.1.2 Microbial Propagation

The objective of propagation is to increase the biomass and consequently the postbiotics yield.

Procedure

1. *First Inoculation*: The activated cultures are placed in a 100 mL bottle containing 90 mL MRS broth and, if necessary, other required nutrients (*see* **Note 5**).
2. *Incubation*: The bottle is incubated for a specific period of time (*see* **Note 6**) at 32–37 °C.

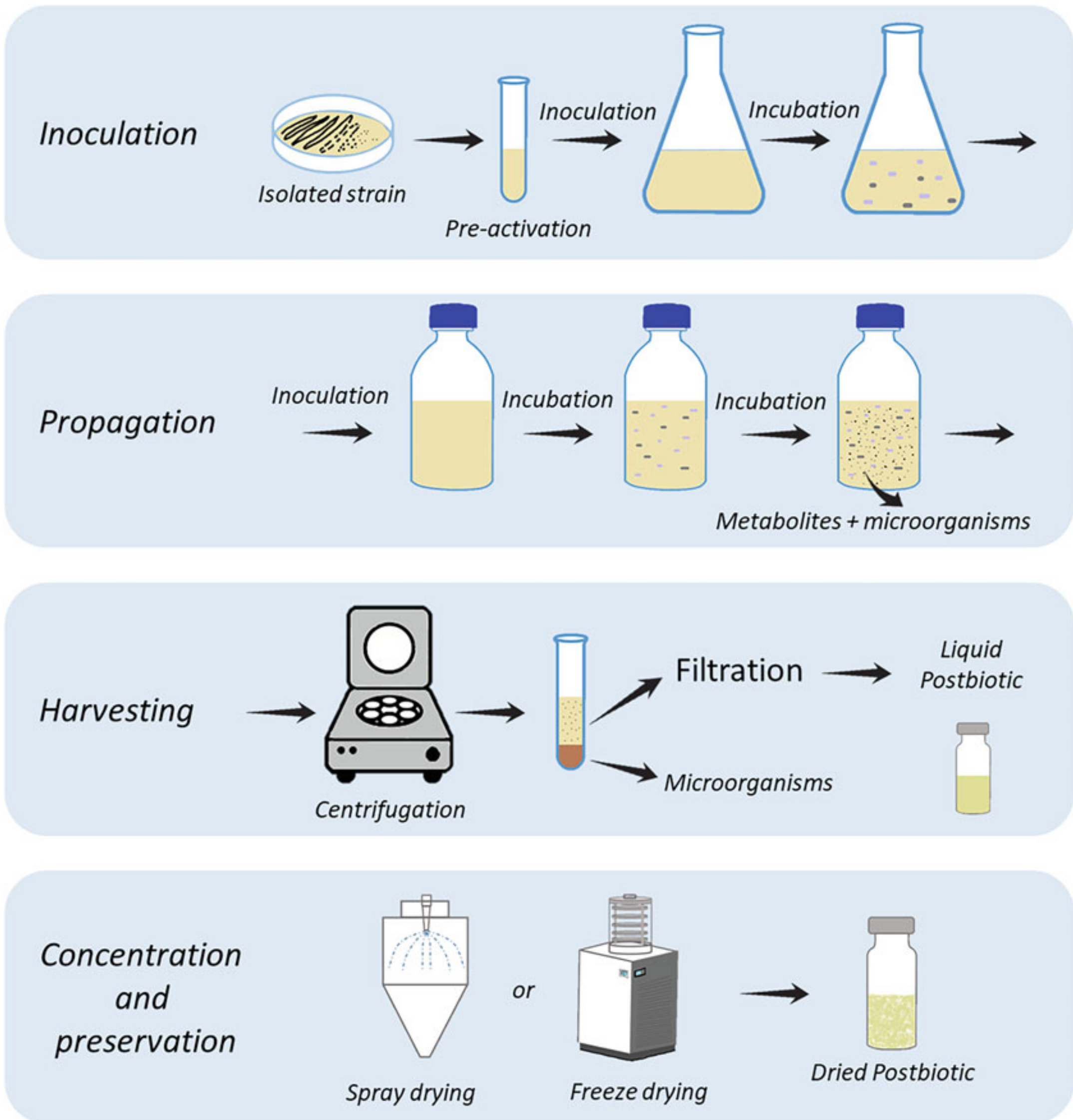

Fig. 2 Schematic procedures of postbiotics (cell-free supernatant) preparation. (Adapted from Moradi et al. [7])

3. *Second Inoculation*: the grown culture is placed in a 1 L MRS broth bottle.
4. *Incubation*: The bottle is incubated for a specific period of time (*see* **Note 6**) at 32–37 ° C.

These **steps** (**1–4**) are continuously repeated until enough probiotic biomass is acquired (*see* **Note** 7).

5. *Post-propagation treatments*: In order to increase the postbiotics yield or to extract specific postbiotics, the microorganisms can be inactivated by the application of post-propagation

conventional treatments (e.g., enzymatic and thermal) or treatments based on emerging technologies (e.g. ultrasound and high-pressure treatments, pulse electric field, among others), then, extracting more metabolites from bacterial cells. Therefore, these treatments can add new intracellular metabolites providing new functionality to the obtained postbiotic [7]. Additional steps may be necessary to eliminate intact cells and recover only the postbiotic fraction such as microfiltration (*see* **Note 8**). Meanwhile, for postbiotics secreted by viable cells, the methods or technology necessary for their recovery from supernatants consist basically of eliminating the viable cells from the medium by centrifugation and/or filtration [9], as described in the Subheading 3.1.3.

3.1.3 Harvesting

The harvesting phase consists in obtaining the desired postbiotics, and separating them from the living microorganisms. This technique is usually the same used for the separation and obtainment of probiotic biomass, but the product will change and additional steps must be carried out to ensure the presence of only postbiotics. There are different ways to extract postbiotics. The most used is the separation of these bioactive extracellular metabolites of the producing cells by centrifugation and filtration techniques [20]. The cell-free supernatants containing the active metabolites can be obtained after the incubation period.

Procedure

1. *Centrifugation*: To extract the cells from the media a cleaning procedure is required. First, the media should be centrifuged at 7000 g for 20 min at 4 °C.
2. *Supernatant separation*: At this moment heavier microorganisms are sent to the bottom of the flasks, then, the supernatant can be separated from the living microorganisms using an electric pipette gun and serological pipette tip (*see* **Note 9**).
   (a) If the purpose is to obtain the microbial cells, the precipitate is washed with 0.9% (v/v) NaCl solution or PBS three times, repeating the procedure, until no MRS broth remains.
   (b) In the case of obtaining postbiotics, the living microorganisms (the bottom part) will be discarded and the supernatant, rich in microbial metabolites, obtained for further procedures.
3. *Filtration*: Finally, the resulting supernatant is filtered using a syringe filter (0.22 to 0.45-μm pore size) to ensure sterility and the absence of microbial cells [21, 22]

3.1.4 Concentration and Preservation Methods

After centrifugation and filtration, the obtained liquid, rich in postbiotics, can be preserved under refrigerated conditions until its application on the food; however, for a longer conservation period and to improve usability in the application, the postbiotic mixture (cell-free supernatant) can be dried by spray drying or freeze drying methods [7, 23]. It must be taken into account that some methods may negatively affect postbiotics functionality (*see* **Note 10**).

Freeze Drying

This is the common method of concentration and preservation for postbiotics used in the literature. Freeze-drying consists of three steps, as described below.

Procedure

1. *Freezing*: ice crystals are formed in postbiotics solution at a temperature – 40 °C under atmospheric pressure within a few hours.
2. *Primary drying*: the pressure is reduced below the equilibrium vapor pressure of ice (pump pressure: 100 mTorr and shelf temperature: – 60 °C).
3. *Secondary drying*: unfrozen water was diffused and desorpted in the postbiotics [23, 24].
4. *Storage*: The obtained dried postbiotics should be kept at 4 °C in polyethylene bags for further use (*see* **Note 11**) and analysis.

Spray Drying

Procedure

Spray drying has been proposed as a low-cost alternative to freeze-drying for the preparation of postbiotics powder.

1. *Evaporation*: water is partially evaporated from postbiotics solution by a rotary evaporator (*see* **Note 12**).
2. *Spray drying*: The postbiotic solution is heated to 37 °C and spray dried under these conditions: inlet air temperature, 119 °C, feeding by a two-fluid nozzle, outlet air temperature 64 °C, and flux of feeding, 6 mL/min (*see* **Note 13**).
3. *Storage*: The obtained dried postbiotics should be kept at 4 °C until use in polyethylene bags for further use and analysis.

3.1.5 Method of Application

After postbiotics preparation, they can be applied to several foods (Fig. 3); however, depending on the food characteristics and how the postbiotic was extracted and preserved, the method of application may also change. The main concerns about postbiotics application to food are the physical-chemical changes that may occur after this addition since some postbiotic mixtures present high moisture, a brown or yellow color, or different sensory attributes

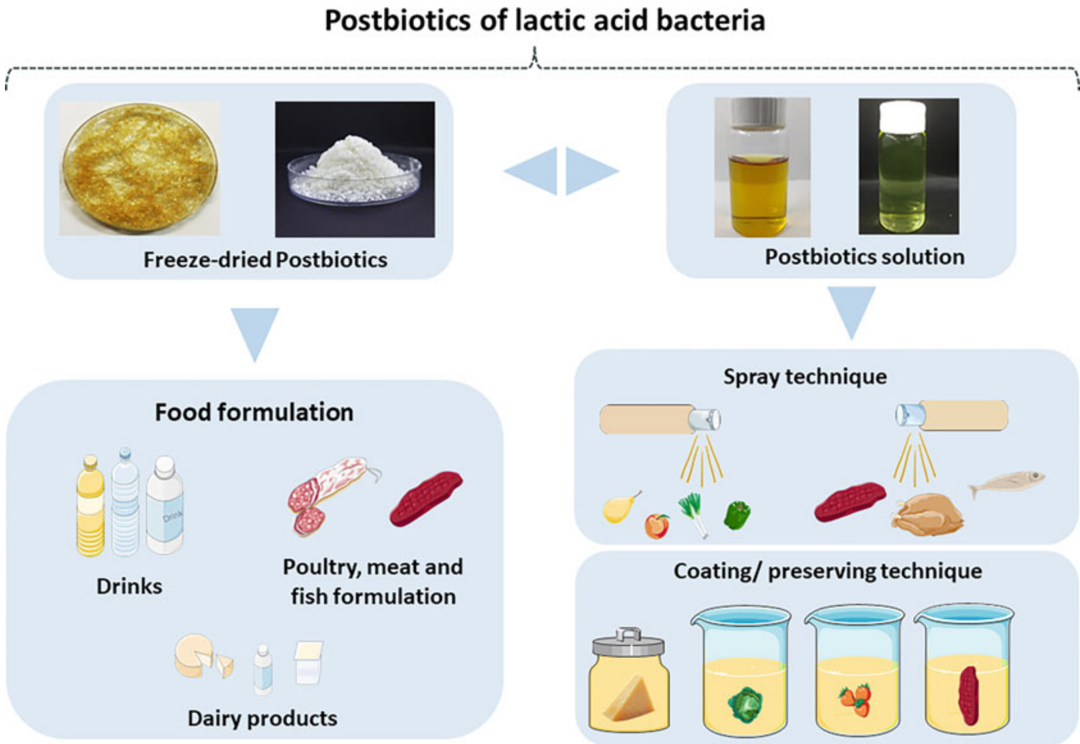

Fig. 3 Methods of postbiotics application in different kinds of food

[7]. To overcome the color changes, alternative media such as a dairy- (buttermilk, whey, and permeate solution) or plant-based solution may help postbiotics to have a more creamy or white appearance; however, reducing the contact time between postbiotics and food may solve the color issue, in some cases. In most cases, postbiotics solution contains proteolytic and lipolytic enzymes, boosting gradually food proteolysis and lipolysis and increasing some food quality index such as thiobarbituric acid (TBA) and total volatile basic nitrogen (TVB-N) levels after postbiotics addition. In this case, enzymatic inactivation of postbiotics is recommended to overcome this drawback. Generally, it is clear that a greater concentration of postbiotics is needed to achieve the same effect in food than in vitro conditions. The ratio varies from 10 to 100 times higher than in the laboratory condition. It varies according to the type of postbiotics, type of food, and method of application.

Depending on the food type, postbiotics can be used by direct addition to food formulation, coating, and spray methods. For example, in fish and meat ground, cheese, bakery, and liquid food, postbiotics in dry form can be applied in free or encapsulated forms in food formulation, while in fish and meat fillets, poultry, fruits, and vegetables spray and coating of postbiotics solutions may be practical. Moreover, the postbiotics solution may be used as a

preserving solution in certain food such as mozzarella cheese. For coating and preservation applications, it is recommended to select a microbial strain with high exopolysaccharides producing feature to produce viscous postbiotics solutions or add postbiotics to other viscous polymer solutions (e.g., chitosan, alginate, etc.), which can improve the rheology and texture of the receiving food. In special cases, postbiotics can be included in food packaging material in order to prepare functional packaging materials to extend shelf life and inhibit surface pathogens.

It is worth mentioning that the chemical composition of each postbiotics solution or powder should be clarified before food application. Since there are many compounds with different natures, there is not a fully clarified method for analyzing the composition of postbiotics. Several chromatographic (e.g., GC, HPLC) methods have been used for the identification of postbiotics constituents. For quantification of phenolics, flavonoids, organic acids, etc., HPLC is the method of choice, while for volatiles and fatty acids, gas chromatography-mass spectrometry is preferred. Additionally, for a complete evaluation, the choice of a proper initial preparation procedure and the use of more than one analytical instrument/procedure is recommended [7, 25].

3.2 Postbiotic Compounds Identification

3.2.1 Vitamins

Vitamins are organic molecules that are supplemented in the diet in a small amount to facilitate various biological processes in the body. Most B-complex group vitamins are directly involved as coenzymes in several energy metabolism reactions. Humans are incapable of biosynthesizing most of the vitamins, and therefore they subsequently have to be supplemented exogenously. Most of the vitamins have to be supplemented through the diet (vitamin A, D, E, etc.); however, limited vitamins (folic acid-B9, cobalamin-B12, Ribofavin-B2) are even synthesized by commensal gut bacteria and some probiotic bacteria [26]. LAB is capable of producing other vitamins in a few quantities. Therefore, postbiotics production can also be directed to the production of important vitamins.

Separation Procedure

Separation begins with selecting a proper extraction method, which varies and depends on the vitamin nature. Similar to food, enzyme extraction from CFS can be done by acid/alkaline hydrolysis, solvent extraction, and solid phase extraction. Folate (vitamin B_9) and riboflavin (B_2) have similar separation procedures with minor differences.

1. *Adding extraction buffer*:
   (a) Folate: extraction buffer [0.1 M phosphate buffer pH 6.8 + 0.5–1.5% (w/v) ascorbic acid (*see* **Note 14**), to avoid vitamin oxidation and degradation] is added to the CFS solution.

   (b) Vitamin B_{12}: 0.57 M phosphate buffer pH 4.5 + 0.05% potassium cyanide solution is added to the CFS solution.

2. *First Centrifugation*: the solution is centrifuged at 5000 g for 10 min.
3. **Heating** (*see* **Note 15**):
   (a) Folate: boiling for 5–15 min.
   (b) Vitamin B_{12}: heated at 100 °C for 30 min.
4. *Second Centrifugation*: the heated solution is centrifuged at 1000 g for 10 min.
5. *Filtration*: the obtained supernatant is filtered with syringe filter (0.45-μm).
6. *Storage*: kept at −70 °C for further analysis.
7. *Identification and quantification:*
   (a) Folate: ELISA, enzymatic and microbiological assays (*see* **Note 16**), (ultra) high-performance liquid chromatography (U) HPLC, or LC-MS [27–29].
   (b) Vitamin B_{12}:: microbiological assay (using *L. delbrueckii* subsp. *lactis* ATCC 7830 as an indicator organism) or available chromatographic methods [30]. Solid phase extraction using C18 cartilages may be also used for the separation of vitamin B_{12} [31].

3.2.2 Short-Chain Fatty Acids

Short-chain fatty acids (SCFAs), comprising 1 to 6 carbon-based anions can be produced during bacterial fermentation, of which acetate (C2), propionate (C3), and butyrate (C4) are the most abundant [32]. SCFAs production tends to participate in preserving the gut barrier function, contributing to the metabolism of carbohydrates and lipids, and act in distinct processes in other tissues, such as adipose tissue remodeling and the immune system. Due to their involvement in energy and lipids metabolism, these molecules can contribute to the reduction of inflammatory risk diseases, obesity, diabetes, and other metabolic failures [33]. Furthermore, they may be responsible for several of the beneficial effects related to the commensal or probiotic bacteria. Propionate, acetate, and butyrate are among the most known SCFAs which are produced by specific microorganisms. The first one is produced commonly by *Lactobacillus* spp. While the others are synthesized by *Bifidobacterium* spp. [34].

Acetate is the most abundant SCFA produced by most enteric bacteria as a fermentation product. Pathways for acetate synthesis are widely distributed among bacterial groups, while pathways for propionate and butyrate appear to be highly conserved and are substrate-specific [35]. Therefore, acetate can be formed by hydrogenotrophic acetogenic bacteria like *Acetobacterium woodii*, from formate through the Wood-Ljungdahl pathway. Acetate is used in part by the bacteria that use them to synthesize some of their structural components [15].

SCFAs are a potential class of postbiotics, the supply of active ingredients at the desired location of the intestine is facilitated; for this reason, diseases located in the intestine have been mostly studied [32]. It is worth mentioning that for SCFAs production, an optimum growth condition (e.g., pH, time, temperature, salt, and SCFA precursors such as glycine, glutamate, threonine, and aspartate) should be used for bacterial culturing depending on the type of strain [34, 36].

Separation Procedure

1. *First Centrifugation*: the postbiotic solution is centrifuged at 3000 *g* for 40 min at room temperature (25 °C).
2. *Supernatant separation*: the supernatant can be separated using a micropipette and transferred into an appropriate vial.
3. *Fatty acids extraction*: The obtained CFS (0.75 mL) is mixed with meta-phosphoric acid (0.3 mL) and the vial is kept at ambient temperature (25 °C) for 25 min to facilitate the fatty acids separation.
4. *Second centrifugation*: The sample is then centrifuged at 5000 g for 15 min
5. *Supernatant separation*: the supernatant containing fatty acids is obtained using a micropipette and the vial stored at −20 °C for further gas chromatography analysis.

3.2.3 Secreted Proteins/Peptides

Enzymes are active proteins that catalyze biochemical reactions [37]. Microbial enzymes possess a variety of biochemical, physiological, and regulatory functions. Industrially, enzymes are derived from a small group of bacterial and fungal strains, mainly *Bacillus subtilis*, *Bacillus licheniformis*, *Aspergillus niger*, and *Aspergillus oryzae* [38]. The main bacteria that produce enzymes of interest are those of the genus *Bacillus*. They are important for their high growth rate that leads to short fermentation times, for their ability to secrete proteins in the extracellular environment, and for being recognized as safe. The *Bacillus* genus is probably the most important bacterial source of proteases. It is capable of producing high yields of neutral and alkaline proteolytic enzymes with remarkable properties, such as high stability against extreme temperatures, pH, organic solvents, detergents, and oxidizing compounds [39]. It has been reported that the maximum production of proteases produced from *Bacillus subtilis* is achieved under the following physical and nutritional aspects: 48 h of incubation time; continuous agitation at 220 rpm, pH 7.5; temperature 45 °C; 2% skim milk; yeast sludge 300 μL; 0.4% ammonium sulphate; urea 0.2%; and cane molasses 0.03% [40].

Among postbiotics generated by microorganisms, there are some peptides with antimicrobial activity, whose mechanism of action is the formation of pores on a bacterial membrane or inhibiting compounds of bacterial wall synthesis [41]. Those peptides can be expressed constitutively or induced in response to infection, as immunomodulatory agents that increase natural innate immunity and as neutralizing agents for endotoxins [42]. The antimicrobial peptides are associated with two interrelated characteristics of the peptides that are their net charge and their propensity to be amphipathic. Both characteristics facilitate their interaction with other bacterial membrane components such as teichoic acids, involving electrostatic interactions between positively charged antimicrobial amino acids and Gram-negative bacterial membrane lipopolysaccharide [15].

Bacteriocins are one of those peptides with a wide antimicrobial activity spectrum, which can be produced by both Gram-positive and Gram-negative bacteria, mainly LAB. Their benefits involve stability, bioengineering, diversity, production, and safety. Nisin is one of the first studied bacteriocins, being considered a class I of antibiotics and reported for many in vitro effects against spoilage and pathogenic bacteria [15]. Bacteriocins have properties to prevent biofilm formation, due to their unique structure. *Pediococcus acidilactici* HW01 releases a pediocin-like bacteriocin (HW01 bacteriocin), which is an efficient antagonist of *Pseudomonas aeruginosa* due to its potential to hinder biofilm progression and production of virulence factors. HW01 bacteriocin is able to attenuate *P. aeruginosa* KCTC 2004 biofilm formation independently of cell death [4].

As a postbiotic, bacteriocins have three main mechanisms to combat biofilm: (a) suppression of twitching motility; this ability of biofilm is mediated by pili, whereas swimming and swarming are the results of flagella activity, (b) disturbing quorum sensing (QS); it alters cell interactions, colonization, and loss of QS signals, (c) reduction of virulence factors (as pyocyanin, protease, and rhamnolipid); pyocyanin contributes to biofilm formation and exposing infection and rhamnolipid from *P. aeruginosa* is also responsible for the conservation of biofilm channels [43]. Alternatively, pre-coating surfaces with bacteriocin from *Limosilactobacillus fermentum* alone is more effective to inhibit *P. aeruginosa* PAO1 biofilm than its simultaneous use with the living bacteria. In addition to cell death, *L. fermentum* bacteriocin causes microenvironment alteration, disorder in cell communication and assembly, decreased QS signals, and weakened cell membrane integrity by pore formation [44].

Separation Procedure

Peptides, bacteriocins, and enzymes have similar separation procedures. Generally, bacteriocins, bacteriocins-like substances (BLS), and peptides are separated, concentrated, and purified by a combination of precipitation-centrifugation-dialysis and chromatographic and electrophoretic techniques for antimicrobial tests (*see* **Note 17**).

1. *Neutralization*: to neutralize the effect of organic acids and hydrogen peroxide, pH is adjusted to 6.2 with NaOH 1 N and 130 U/mL of catalase is added.
2. *Precipitation*: The process is started by adding ammonium sulfate (*see* **Note 18**) to reach the desired saturation percentage (70%) (*see* **Note 19**)
3. *Mixing*: the solution is mixed with a magnetic stirrer at 4 °C for 6–8 h.
4. *Centrifugation*: the mixture is centrifuged (10,000 g for 20 min at 4 °C) to obtain protein precipitant containing BLS.
5. *Dialysis*: A small volume of 10 mM PBS (or distilled water) at pH 2.5 is added to the sample and dialyzed for 8 h at 4 °C against PBS (pH 7) in dialysis tubing or dialysis cassettes (1 kDa cut-off) (*see* **Note 20**) with two changes of buffer (the ratio of the postbiotics to dialysis solution is 1: 500).
6. *Identification and characterization*: The obtained solution is referred to as partially purified bacteriocin and can be used for antimicrobial tests; however, further steps using chromatographic methods (Ion exchange Sephadex G-25 column and reverse-phase HPLC), sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), amino acid sequence and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) can be applied to purify and characterize a specific bacteriocin from postbiotics [22, 45–47].

4 Notes

1. Other media could be chosen depending on strain growth performance, the desired postbiotics functionality, which can be affected by the type of medium and culture condition. For example, postbiotics prepared in tryptic soy broth reveal higher antioxidant capacity than those prepared in MRS [14].
2. Follow one of these procedures for activation depending on the nature of desired strain:
   (a) Transfer 3–5 colonies of a bacterium from agar plate to the culture broth.

   (b) Transfer a frozen solution of the bacterium to a culture broth (1 to 100 ratio).
   (c) Add an appropriate amount of commercial lyophilized strains according to the company's instruction

3. In most cases, lithium chloride (0.1% w/v) is added to prevent the growth of Gram-negative bacteria. To ensure the source of nitrogen, L-cysteine (0.05% w/v is also added to the MRS medium [48].

4. To adjust the turbidity of bacterial suspension, use one of these methods:
   (a) McFarland standards: Prepare or buy the desired McFarland Equivalence Turbidity Standards (0.5, 1.0, 2.0, 3.0, 4.0)
   (b) Use commercial McFarland Densitometer
   (c) Spectrophotometric method: Adjust the optical density of bacterial suspension to a level where the exact bacterial population in it is already determined. Then to confirm and ensure the count, prepare different dilutions, culture, and enumerate according to the common bacterial enumeration method. The bacterial suspension should not be kept in PBS for more than 15 min at room temperature. However, keeping the bacteria for more than 15 min causes cell lysis and its inoculation should be avoided.

5. The medium composition should be balanced between precursors and other additives (carbon and nitrogen source, cofactors, or polysorbates), since some precursors may improve the synthesis of postbiotics by indirect overstimulation of other biosynthetic routes [34].

6. The incubation time will depend on the strain growth rate and metabolism.

7. At an industrial level, reactors can be used to produce large amounts of microbial biomass in less time. It is important to mention that after each growth phase, a counting analysis should be done to assess the microbial concentration and then establish a production process for microbial biomass obtainment.

8. In general, the loss of viability from living microorganisms occurs after exposure to factors that alter microbial cell structure and/or change their physiological functions without disrupting the bacterial membrane and cell wall necessary to maintain the cell structure intact. The viability may also decrease by disrupting the bacterial membrane with the application of at least two treatments in order to obtain the intracellular metabolites and/or cell wall components in the form of fragments.

9. Transfer CFS as quickly as possible to a new container to avoid resuspending bacterial cell in the CFS [22].
10. For example, during spray drying, certain volatile metabolites are lost or lyophilization removes hydrogen peroxide from postbiotics solution [7]. Moreover, the antioxidant activity of lyophilized postbiotics of some LAB is sevenfold lower than the initial postbiotics solution (Unpublished observations).
11. It is recommended to be used on the day of preparation.
12. As an alternative to increasing the total solids, maltodextrin (20–40%) needs to be added to postbiotics solution [49].
13. Lactic acid in postbiotics is very hygroscopic and thermoplastic acid makes the drying process very difficult [49].
14. For riboflavin separation, acetic acid (1% v/v) is commonly added instead of buffer with ascorbic acid [29].
15. Vitamins must be protected from oxidation by performing the extraction under dim light and cooling the samples in ice after heating.
16. In this test, an indicator microorganism (*L. casei* subsp. *rhamnosus* NCIMB 10463 or *L. casei* NCIM 2364) is used for quantification of folate based on the AOAC official methods for folate quantification using available folic acid test kit according to the manufacturer's instructions.
17. As an alternative, the organic solvent (ethanol or acetone) extraction technique can be used for the separation of some bacteriocins. In this method, postbiotics is exposed to three volumes of cold acetone at –30 °C [50].
18. This is an effective chemical used commonly for peptides precipitation without affecting bioactivity [22].
19. Some bacteriocins may precipitate at lower concentrations of ammonium sulphate, then it is important to determine what level of salt precipitates the peptide of interest [50]
20. A dialysis bag should be carefully selected as most bacteriocins have a size smaller than 10,000 Da. For separation of peptides with a size below 10 KDs, CFS is purified using 10 kDa ultrafiltration membranes [50].

References

1. Balthazar CF, Guimarães JF, Coutinho NM, Pimentel TC, Ranadheera CS (2022) The future of functional food: emerging technologies application on prebiotics, probiotics and postbiotics. Compr Rev Food Sci Food Saf 21(3):2560–2586. https://doi.org/10.1111/1541-4337.12962
2. Salminen S, Collado MC, Endo A, Hill C, Lebeer S, Quigley EMM et al (2021) The international scientific Association of Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of postbiotics. Nat Rev Gastroenterol Hepatol 18(9):649–667. https://doi.org/10.1038/s41575-021-00440-6
3. Tsilingiri K, Rescigno M (2013) Postbiotics: what else? Benefic Microbes 4(1):101–107. https://doi.org/10.3920/bm2012.0046

4. Moradi M, Kousheh SA, Almasi H, Alizadeh A, Guimarães JT, Yılmaz N et al (2020) Postbiotics produced by lactic acid bacteria: the next frontier in food safety. Compr Rev Food Sci Food Saf 19(6):3390–3415. https://doi.org/10.1111/1541-4337.12613
5. Vinderola G, Sanders ME, Salminen S (2022) The concept of postbiotics. Foods 11(8):1077
6. Bourebaba Y, Marycz K, Mularczyk M, Bourebaba L (2022) Postbiotics as potential new therapeutic agents for metabolic disorders management. Biomed Pharmacother 153: 113138. https://doi.org/10.1016/j.biopha.2022.113138
7. Moradi M, Molaei R, Guimarães JT (2021) A review on preparation and chemical analysis of postbiotics from lactic acid bacteria. Enzym Microb Technol 143:109722. https://doi.org/10.1016/j.enzmictec.2020.109722
8. Vallejo-Cordoba B, Castro-López C, García HS, González-Córdova AF, Hernández-Mendoza A (2020) Chapter one: postbiotics and paraprobiotics: a review of current evidence and emerging trends. In: da Cruz AG, Prudencio ES, Esmerino EA, da Silva MC (eds) Advances in food and nutrition research. Academic Press, pp 1–34
9. Cuevas-González PF, Liceaga AM, Aguilar-Toalá JE (2020) Postbiotics and paraprobiotics: from concepts to applications. Food Res Int 136:109502. https://doi.org/10.1016/j.foodres.2020.109502
10. Garnier L, Mounier J, Lê S, Pawtowski A, Pinon N, Camier B et al (2019) Development of antifungal ingredients for dairy products: from in vitro screening to pilot scale application. Food Microbiol 81:97–107. https://doi.org/10.1016/j.fm.2018.11.003
11. Takahashi JA, Barbosa BV, Martins BA, Guirlanda PC, Moura AF (2020) Use of the versatility of fungal metabolism to meet modern demands for healthy aging, functional foods, and sustainability. J Fungi 6(4):223
12. Her J-Y, Kim MS, Lee K-G (2015) Preparation of probiotic powder by the spray freeze-drying method. J Food Eng 150:70–74. https://doi.org/10.1016/j.jfoodeng.2014.10.029
13. Soares MB, Martinez RCR, Pereira EPR, Balthazar CF, Cruz AG, Ranadheera CS et al (2019) The resistance of bacillus, Bifidobacterium, and lactobacillus strains with claimed probiotic properties in different food matrices exposed to simulated gastrointestinal tract conditions. Food Res Int 125:108542. https://doi.org/10.1016/j.foodres.2019.108542
14. İncili GK, Karatepe P, Akgöl M, Güngören A, Koluman A, İlhak Oİ et al (2022) Characterization of lactic acid bacteria postbiotics, evaluation in-vitro antibacterial effect, microbial and chemical quality on chicken drumsticks. Food Microbiol 104:104001. https://doi.org/10.1016/j.fm.2022.104001
15. Hernández-Granados MJ, Franco-Robles E (2020) Postbiotics in human health: possible new functional ingredients? Food Res Int 137: 109660. https://doi.org/10.1016/j.foodres.2020.109660
16. Balthazar CF, Santillo A, Guimarães JT, Capozzi V, Russo P, Caroprese M et al (2019) Novel milk–juice beverage with fermented sheep milk and strawberry (Fragaria × ananassa): nutritional and functional characterization. J Dairy Sci 102:10724. https://doi.org/10.3168/jds.2019-16909
17. Zacarchenco PB, Massaguer-Roig S (2006) Properties of Streptococcus thermophilus fermented milk containing variable concentrations of Bifidobacterium longum and lactobacillus acidophilus. Braz J Microbiol 37: 338–344
18. Dunand E, Burns P, Binetti A, Bergamini C, Peralta G, Forzani L et al (2019) Postbiotics produced at laboratory and industrial level as potential functional food ingredients with the capacity to protect mice against salmonella infection. J Appl Microbiol 127(1):219–229
19. Amiri S, Rezaei Mokarram R, Sowti Khiabani M, Rezazadeh Bari M, Alizadeh KM (2019) Exopolysaccharides production by lactobacillus acidophilus LA5 and Bifidobacterium animalis subsp. lactis BB12: optimization of fermentation variables and characterization of structure and bioactivities. Int J Biol Macromol 123:752–765. https://doi.org/10.1016/j.ijbiomac.2018.11.084
20. Dinić M, Lukić J, Djokić J, Milenković M, Strahinić I, Golić N et al (2017) Lactobacillus fermentum postbiotic-induced autophagy as potential approach for treatment of acetaminophen hepatotoxicity. Front Microbiol 8:594. https://doi.org/10.3389/fmicb.2017.00594
21. Żółkiewicz J, Marzec A, Ruszczyński M (2020) Postbiotics-a step beyond pre- and probiotics. Nutrients 12:8. https://doi.org/10.3390/nu12082189
22. Twomey E, Hill C, Field D, Begley M (2021) Recipe for success: suggestions and recommendations for the isolation and characterisation of Bacteriocins. Int J Microbiol 2021:9990635. https://doi.org/10.1155/2021/9990635
23. Moradi M, Tajik H, Mardani K, Ezati P (2019) Efficacy of lyophilized cell-free supernatant of lactobacillus salivarius (ls-BU2) on Escherichia coli and shelf life of ground beef. Vet Res

Forum 10(3):193–198. https://doi.org/10.30466/vrf.2019.101419.2417

24. Kawasaki H, Shimanouchi T, Kimura Y (2019) Recent development of optimization of Lyophilization process. J Chem 2019: 9502856. https://doi.org/10.1155/2019/9502856
25. İncili GK, Karatepe P, Akgöl M, Tekin A, Kanmaz H, Kaya B et al (2022) Impact of chitosan embedded with postbiotics from Pediococcus acidilactici against emerging foodborne pathogens in vacuum-packaged frankfurters during refrigerated storage. Meat Sci 188:108786. https://doi.org/10.1016/j.meatsci.2022.108786
26. Nataraj BH, Ali SA, Behare PV, Yadav H (2020) Postbiotics-parabiotics: the new horizons in microbial biotherapy and functional foods. Microb Cell Factories 19(1):168. https://doi.org/10.1186/s12934-020-01426-w
27. Gangadharan D, Sivaramakrishnan S, Pandey A, Madhavan Nampoothiri KESAVAN (2010) Folate-producing lactic acid bacteria from cow's milk with probiotic characteristics. Int J Dairy Technol 63(3):339–348. https://doi.org/10.1111/j.1471-0307.2010.00590.x
28. Laiño JE, Juárez del Valle M, Hébert EM, Savoy de Giori G, LeBlanc JG (2017) Folate production and fol genes expression by the dairy starter culture Streptococcus thermophilus CRL803 in free and controlled pH batch fermentations. LWT Food Sci Technol 85: 146–150. https://doi.org/10.1016/j.lwt.2017.07.004
29. Cárdenas N, Laiño JE, Delgado S, Jiménez E, Juárez del Valle M, Savoy de Giori G et al (2015) Relationships between the genome and some phenotypical properties of lactobacillus fermentum CECT 5716, a probiotic strain isolated from human milk. Appl Microbiol Biotechnol 99(10):4343–4353. https://doi.org/10.1007/s00253-015-6429-0
30. Masuda M, Ide M, Utsumi H, Niiro T, Shimamura Y, Murata M (2012) Production potency of folate, vitamin B12, and thiamine by lactic acid bacteria isolated from Japanese pickles. Biosci Biotechnol Biochem 76(11): 2061–2067. https://doi.org/10.1271/bbb.120414
31. Li P, Gu Q, Yang L, Yu Y, Wang Y (2017) Characterization of extracellular vitamin B12 producing lactobacillus plantarum strains and assessment of the probiotic potentials. Food Chem 234:494–501. https://doi.org/10.1016/j.foodchem.2017.05.037
32. Gill PA, van Zelm MC (2018) Review article: short chain fatty acids as potential therapeutic agents in human gastrointestinal and inflammatory disorders. Aliment Pharmacol Ther 48(1):15–34. https://doi.org/10.1111/apt.14689
33. Ríos-Covián D, Ruas-Madiedo P, Margolles A, Gueimonde M, de Los Reyes-Gavilán CG, Salazar N (2016) Intestinal short chain fatty acids and their link with diet and human health. Front Microbiol 7:185. https://doi.org/10.3389/fmicb.2016.00185
34. Chávarri M, Diez-Gutiérrez L, Marañón I, Javier R, Barron L (2021) Chapter 17: secondary metabolites from probiotic metabolism. In: Dhanasekaran D, Sankaranarayanan A (eds) Advances in probiotics. Academic Press, pp 259–276
35. Morrison DJ, Preston T (2016) Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut Microbes 7(3):189–200. https://doi.org/10.1080/19490976.2015.1134082
36. Hadinia N, Edalatian Dovom MR, Yavarmanesh M (2022) The effect of fermentation conditions (temperature, salt concentration, and pH) with lactobacillus strains for producing short chain fatty acids. LWT 165:113709. https://doi.org/10.1016/j.lwt.2022.113709
37. Singhania RR, Patel AK, Thomas L, Goswami M, Giri BS, Pandey A (2015) Industrial enzymes. Industrial biorefineries & white biotechnology. Elsevier, pp 473–497
38. Kumar V, Baweja M, Liu H, Shukla P (2017) Microbial enzyme engineering: applications and perspectives. Recent advances in applied microbiology. Springer, pp 259–273
39. Contesini FJ, Melo RR, Sato HH (2018) An overview of bacillus proteases: from production to application. Crit Rev Biotechnol 38(3): 321–334. https://doi.org/10.1080/07388551.2017.1354354
40. Butt KY, Altaf A, Malana MA, Ghori MI, Jamil A (2018) Optimal production of proteases from Bacillus subtilis using submerged fermentation. Pak J Life Soc Sci 16(1):15–19
41. Scocchi M, Mardirossian M, Runti G, Benincasa M (2016) Non-membrane permeabilizing modes of action of antimicrobial peptides on bacteria. Curr Top Med Chem 16(1):76–88. https://doi.org/10.2174/1568026615666150703121009
42. Chung PY, Khanum R (2017) Antimicrobial peptides as potential anti-biofilm agents against multidrug-resistant bacteria. J Microbiol Immunol Infect 50(4):405–410. https://doi.org/10.1016/j.jmii.2016.12.005

43. Lee DH, Kim BS, Kang SS (2020) Bacteriocin of pediococcus acidilactici HW01 inhibits biofilm formation and virulence factor production by Pseudomonas aeruginosa. Probiotics Antimicrob Proteins 12(1):73–81. https://doi.org/10.1007/s12602-019-09623-9
44. Sharma V, Harjai K, Shukla G (2018) Effect of bacteriocin and exopolysaccharides isolated from probiotic on P. aeruginosa PAO1 biofilm. Folia Microbiol 63(2):181–190. https://doi.org/10.1007/s12223-017-0545-4
45. Svetoch EA, Eruslanov BV, Perelygin VV, Mitsevich EV, Mitsevich IP, Borzenkov VN et al (2008) Diverse antimicrobial killing by enterococcus faecium E 50-52 Bacteriocin. J Agric Food Chem 56(6):1942–1948. https://doi.org/10.1021/jf073284g
46. Repally A, Perumal V, Dasari A, Palanichamy E, Venkatesan A (2018) Isolation, identification of lactobacillus mucosae AN1 and its Antilisterial peptide purification and characterization. Probiotics Antimicrob Proteins 10(4):775–786. https://doi.org/10.1007/s12602-017-9341-3
47. Saavedra L, Castellano P, Sesma F (2004) Purification of Bacteriocins produced by lactic acid bacteria. In: Spencer JFT, Ragout de Spencer AL (eds) Public health microbiology: methods and protocols. Humana Press, Totowa, NJ, pp 331–336
48. Amiri S, Rezaei Mokarram R, Sowti Khiabani M, Rezazadeh Bari M, Alizadeh KM (2020) In situ production of conjugated linoleic acid by Bifidobacterium lactis BB12 and lactobacillus acidophilus LA5 in milk model medium. LWT 132:109933. https://doi.org/10.1016/j.lwt.2020.109933
49. Burns P, Molinari F, Beccaria A, Páez R, Meinardi C, Reinheimer J et al (2010) Suitability of buttermilk for fermentation with lactobacillus helveticus and production of a functional peptide-enriched powder by spray-drying. J Appl Microbiol 109(4):1370–1378. https://doi.org/10.1111/j.1365-2672.2010.04761.x
50. Pingitore EV, Salvucci E, Sesma F, Nader-Macias M (2007) Different strategies for purification of antimicrobial peptides from lactic acid bacteria (LAB). In Communicating current research and educational topics and trends in applied microbiology, vol 1, pp 557–68

Chapter 17

Psychobiotic Carried by Food and Beverage

Cássia Pereira Barros, Erick A. Esmerino, Roberto Laureano Melo, and Adriano Gomes da Cruz

Abstract

Common mental disorders such as anxiety and depression have increased in prevalence in recent decades, becoming a global health challenge. In this context, it is necessary to search for therapeutic approaches aimed at preventing and treating these disorders. The term psychobiotics refers to beneficial bacteria (probiotics) or support for these bacteria (prebiotics) that influence bacteria–brain relationships. In addition to improving bowel function, they may also exert anxiolytic and antidepressant effects through immunological, humoral, neuronal, and metabolic pathways. Therefore, the use of psychobiotics can help the maintenance and/or restoration of the population's mental health. This chapter describes in detail protocols for the elaboration of a food product with psychobiotic potential. In addition, the most used behavioral tests for preclinical trials that can be applied to confirm the psychobiotic effect are also discussed.

Key words Microbiota, Probiotics, Prebiotics, Mental wellness, Adjunctive therapy, Fermented food

1 Introduction

Common mental disorders such as anxiety and depression have increased substantially in recent decades. According to the World Health Organization, depression affects more than 300 million people, and it is estimated that by 2030 it will be the main cause of disability in the world [1]. Depression is associated with persistent depressed mood, inability to experience pleasure or interest, fatigue, psychomotor retardation, poor concentration, and suicidal thoughts. It is important to emphasize that depressed individuals may simultaneously suffer from anxiety, a condition represented by a state of discomfort, fear, and uncertainty about the future [2, 3]. Therefore, such disorders, in addition to negatively affecting the quality of life of patients, can also cause great economic losses due to the drop in productivity at work and the high costs of treatments for health and well-being.

Adriano Gomes da Cruz et al. (eds.), *Probiotic Foods and Beverages: Technologies and Protocols*, Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-3187-4_17,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2023

Currently, there is a wide range of therapeutic interventions for the treatment of mental disorders. However, these interventions often only relieve symptoms, they take too long to take effect or are ineffective, which can increase the suicide rate. In addition, most of the medications used cause undesirable side effects such as mood swings, sleep disturbances, dependence and addiction, and changes in the health of other parts of the body [4–6]. In this context, there is an urgent need for approaches that are faster, safer, and more effective in restoring and maintaining psychological health.

Among the new therapeutic methodologies, the modification of the intestinal microbiota has attracted much interest due to the bidirectional interaction of the microbiome with the nervous system via the gut–brain axis. Several studies have shown that consumption of beneficial microorganisms can reduce symptoms of depression, anxiety, and stress and improve cognition, suggesting possibilities for a new class of probiotics with psychotropic activity called "psychobiotics" [7, 8]. The emerging term "psychobiotic" was created to define the group of probiotic bacteria that, when ingested in adequate amounts, promote beneficial effects on mental health [9]. Later, this definition was updated to include any exogenous factor capable of exerting actions in the brain that are mediated by bacteria [10]. Therefore, the concept of psychobiotics came to include prebiotics as they contribute to the growth of beneficial intestinal bacteria.

Psychobiotics can be consumed through a healthy diet, dietary supplements, and functional fermented foods. Significant improvements in physical and mental health parameters were reported in clinical trials conducted with petrochemical workers and healthy students under academic stress after consumption of probiotic yogurt and fermented milk, respectively, when compared to the placebo group [11, 12]. Therefore, these findings support that nutritional therapies with psychobiotics can modulate the psychological symptoms associated with intestinal dysfunction.

In this context, animal models are important in evaluating the safety, the efficacy and action mechanism of new drugs [13]. In addition, these models help to understand the neurobiological mechanisms inherent to mental health. Currently, preclinical trials have been considered the backbone of new drug research, having contributed decisively to the development of current treatments for depression and anxiety disorders [14].

Given the above, the objective of this chapter is to provide guidelines for the development of a food supplemented with probiotic bacteria with psychobiotic potential. In addition, the behavioral tests most used for preclinical trials that can be conducted to confirm the psychobiotic effect of the food product are also described.

2 General Recommendations Proposed for Application of Psychobiotic Strains in Foods and Beverages

It is advisable to start with the following criteria:

1. Selection of strain(s) – first, a literature search must be carried out in order to investigate microorganisms that have already been identified as probiotics and that, therefore, have been previously proven in preclinical and clinical trials in humans which, in addition to providing beneficial effects on the host, are safe and capable of transiently surviving through the gastrointestinal tract, adhering to intestinal epithelial cells and resisting intestinal acidity, bile, and high-count foods. In addition, it is possible to choose formulations with single microbial strains or multi-strain formulations containing two or more strains. Then, choose those that have already been studied for the desired psychobiotic effect (Table 1), since not all probiotics share the same mechanisms of action and clinical benefits. Otherwise, it is possible to perform a rapid screening to assess whether the chosen strain has psychobiotic activity through in vitro tests. In general, beneficial bacteria with psychobiotic characteristics are represented by the genera *Lactobacillus*, *Bifidobacterium*, and *Lactococcus* [15]. On the other hand, it should be noted that recently several species of microorganisms allocated to the genus *Lactobacillus* were reclassified after evaluating their phenotypic, genotypic, and ecological characteristics in 24 new genera. Among them, *Lacticaseibacillus*, *Lactiplantibacillus*, *Limosilactobacillus* and *Levilactobacillus* have psychobiotic properties.
2. Determination of microorganism concentration, frequency, and time of consumption – the cell density of the psychobiotic microorganism normally ranges from 10^9 to 10^{11} CFU, with a consumption frequency of once or twice a day for about 2 to 8 weeks [16, 17].
3. Selection of the food matrix – fermented foods have been used as psychobiotic delivery vehicles. They are defined as foods and beverages obtained through enzymatic conversions of food components mediated by microorganisms. In this context, they are considered an excellent source of probiotics due to the large amount of lactic acid bacteria naturally present in the raw material or intentionally added as starter cultures to preserve and improve the sensory characteristics of the product [18, 19]. In fact, the psychobiotic effect was first reported after daily consumption of a dairy drink containing the probiotic *Lactobacillus casei* Shirota caused a general improvement in mood [20]. Subsequently, further research was carried out with fermented products based on milk (yoghurt, kefir, milk),

Table 1
Examples of bacteria strains with psychobiotic potential

Psychobiotic Effects	Gut Microbes	Psychobiotic Strains
Anxiolytic activity	*Lactobacillus* spp.	*L. acidophilus* LA5 *L. helveticus* ROO52 *L. helveticus* NS8
	Lactiplantibacillus spp.	*L. plantarum* DR7
	Lacticaseibacillus spp.	*L. casei* Shirota *L. rhamnosus* JB-1
	Limosilactobacillus spp.	*L. fermentum* NS9
	Bifidobacterium spp.	*B. breve* 1205 *B. breve* CCFM1025 *B. longum* 1714 *B. longum* NCC3001 *B. longum* R0175
Antidepressant activity	*Lactobacillus* spp.	*L. acidophilus* LA5 *L. acidophilus* W37 *L. gasseri* OLL2809 *L. helveticus* NS8
	Levilactobacillus spp.	*L. brevis* W63
	Lactiplantibacillus spp.	*L. plantarum* 90sk
	Lacticaseibacillus spp.	*L. casei* Shirota *L. casei* W56
	Lactococcus spp.	*L. lactis* W19 *L. lactis* W58
	Bifidobacterium spp.	*B. bifidum* W23 *B. breve* M2CF22M7 *B. breve* CCFM1025 *B. infantis* 35624 *B. lactis* W52 *B. longum* R0175 *B. longum* subsp. *infantis* E41
Anti-stress activity	*Lactobacillus* spp.	*L. acidophilus* LA5 *L. gasseri* CP2305 *L. helveticus* R0052
	Lacticasebacillus spp.	*L. casei* Shirota *L. rhamnosus* JB-1
	Bifidobacterium spp.	*B. breve* CCFM1025 *B. logum* R0175

soy (milk, paste, sauce, tempeh or natto), rice (bran), algae (*Laminaria japonica*, *Saccharina japonica*), black carrots (aqueous extract), and green or black teas and sucrose solution (kombucha) with the aim of investigating any biological activity they could exert on the central nervous system [21]. However, it is important to highlight that each fermented food has specific effects that can change according to the chosen food matrix, type of fermentation used or microorganisms involved [19]. Dairy-based matrix are considered ideal for proliferation

of probiotics because they contain a large amount of carbon and essential amino acids resulting from the hydrolysis of lactose and the proteolytic system involved in the use of casein. Additionally, the buffering capacity of milk and its fat content provide a suitable condition for probiotics to better tolerate the adverse conditions of the gastrointestinal tract [22]. Table 2 includes several studies that show the potential psychobiotic applications in fermented food products. In general, it was possible to observe that most of these trials, whether in vivo murine model or clinical trial, involved the use of fermented milk as the main carrier matrix of psychobiotics of the genus *Lactobacillus* spp. present in concentrations between 10^6 and 10^{10} CFU /g/mL in the food consumed daily in varying dosages for a period between 4 and 12 weeks.

3 General Considerations for the Evaluation of Psychobiotic Activity in the Food Product by Behavior Tests

Behavioral assessment is an important tool to assess the actions of psychobiotics. However, some considerations are essential to guarantee the reliability and reproducibility of the results obtained. In principle, the adoption of the principles of use and care of laboratory animals are important to avoid or minimize discomfort, distress, and pain. Thus, animals must be kept in environments where there is control of temperature and humidity, adequate ventilation and air quality, as well as light cycle and noise control. All recommendations to ensure an adequate macroenvironment for carrying out the experimental protocols are present in the federal laboratory animal regulations and local institutional rules.

In addition to a properly controlled macroenvironment, it is necessary to pay attention to the animal model to be chosen to evaluate the action of psychobiotics. Although porcine and zebrafish models have become more prevalent, rats and mice are the most commonly used animals for behavioral assessment. Mice, in particular, are more useful due of their ability to reproduce and mature rapidly, and the relative ease to which genetic modification can be applied through mutational, transgenic, and knockout approaches. Once the animal model has been chosen, the gender, age and strain must be standardized. It may seem like an excess of care, but there are numerous scientific studies that demonstrate that these factors can interfere with the stress response and, consequently, anxiety-like behavior. For example, females show less general activity on tests to assess anxiety, such as the elevated plus maze [42].

Table 2
Psychobiotic strains' applications in fermented foods products

Fermented Food	Psychobiotic Strain(s)/ Concentration	Model	Dose/Treatment duration	Outcomes	References
Black carrot	*Lactiplantibacillus plantarum* SRCM 9	In vivo murine model	2% fermented black carrot with *L. plantarum* for 8 weeks	Better cognitive function by preventing hippocampal insulin resistance associated with lower amyloid-β deposition in type 2 diabetic rats with dementia	[23]
Black soybean milk	*Levilactobacillus brevis* FPA 3709 (1×10^6 CFU/mL)	In vivo murine model	35 mg/kg or 70 mg/kg body weight by oral gavage for 28 days	Antidepressant effect without side effects in rat models	[24]
GABA-rich fermented milk	*Levilactobacillus brevis* DL-11 (10^8 CFU/mL)	In vivo murine model	8.83, 16.67 and 33.33 mg/kgbody weight / day for 4 weeks	Relieved anxiety and improved sleep quality, that may be associated with significant increases of SCFAs in the intestine and related to changes in the composition of the gut microbiota in mice	[25]
Milk	*Lactobacillus casei* Shirota (1×10^8 CFU/mL)	Clinical trial	65 mL for 3 weeks	General improved the mood of adults whose mood was initially poor/ depressive	[20]
Milk	*Bifidobacterium animalis* subsp. *lactis* I-2494 (1.25×10^{10} CFU/ dose), *Streptococcus thermophilus* I-1630, *Lactobacillus delbrueckii* subs. *bulgaricus* I-1632 and I-1519 (1.2×10^9 CFU/125 g) and *Lactococcus lactis* subsp. *lactis* (1.2×10^9 CFU/125 g).	Clinical trial	125 g twice daily for 4 weeks	Affected the activity of brain regions that control central processing of emotion and sensation in healthy women	[26]
Milk	*Lactobacillus helveticus* IDCC3801	Clinical trial	500, 1000, or 2000 mg of tablets of skim milk powder fermented for 12 weeks	Improved cognitive function in healthy old adults	[27]

Milk	*Lactobacillus acidophilus*, *Lacticaseibacillus casei*, *Bifidobacterium bifidum*, and *Limosilactobacillus fermentum* (2×10^9 CFU/g for each)	Clinical trial	200 mL/day for 12 weeks	Positively affected cognitive function and some metabolic statuses in Alzheimer's disease patients (60–95 years old)	[28]
Milk	*Lacticaseibacillus casei Shirota* YIT 9029 (1×10^9 CFU/mL)	Clinical trial and In vivo murine model	100 mL/day for 8 weeks	Improved symptoms of stressed subjects	[12]
Milk	*Limosilactobacillus fermentum* LAB9 (10^9 CFU/0.2 mL) or *L. casei* LABPC (10^9 CFU/0.2 mL)	In vivo murine model	0.2 mL/day by oral gavage for 4 weeks	There was a restoration of cholinergic neurotransmission and attenuation of neuroinflammation in mice	[29]
Milk	*Lactobacillus gasseri* CP2305 (1×10^{10} CFU / 190 g)	Clinical trial	190 g/day for 5 weeks	Improvement in sleep quality and alleviated stress-associated symptoms in healthy students	[30]
Milk	*Lactobacillus gasseri* CP2305 (1×10^{10} CFU/2,5 g)	Clinical trial	2,5 g of powder dissolved in water/day for 4 weeks	Improved physical and mental states in students in the cadaver dissection course	[31]
Milk drink containing lactononadecapeptide	*Lactobacillus helveticus* CM4	Clinical trial	190 g/day for 8 weeks	Improvement in attention and memory in healthy middle-aged adults	[32]
Laminaria japonica	*Levilactobacillus brevis* BJ20	Clinical trial	1.5 g/day for 6 weeks	Demonstrated a protective mechanism against cognitive impairment associated with dementia in elderly	[33]
Saccharina japonica algae	*Levilactobacillus* BJ20	Clinical trial	500 mg/twice daily for 4 weeks	Changes in memory ability via regulation of SOD antioxidant system	[34]
Soybean	*Lactiplantibacillus plantarum* C29 (1.25×10^{10} CFU/g)	Clinical trial	800 mg/day for 12 weeks		[35]

(continued)

Table 2 (continued)

Fermented Food	Psychobiotic Strain(s)/ Concentration	Model	Dose/Treatment duration	Outcomes	References
				Improved cognitive function in individuals with mild cognitive impairment	
Rice bran	*Saccharomyces cerevisiae* IFO 2346	In vivo murine model	1 g/kg/day of a hot water extract of fermented rice bran for 2 weeks	Provided anti-stress and anti-fatigue effects in rats and mice	[36]
Kefir – fermented Milk	4% kefir grains containing *Acetobacter aceti*, *Acetobacter* sp., *Lactobacillus delbrueckii*, *Limosilactobacillus fermentum*, *Fructilactobacillus fructivorans*, *enterococcus faecium*, *Leuconostoc* spp., *Lactobacillus kefiranofaciens*, *Candida famata*, and *Candida krusei*	Clinical trial	2 mL/kg/day for 90 days	Improved memory, visual-spatial/ abstraction abilities, and executive/ language functions	[37]
Kefir – fermented Milk	Kefir grains dominated by *Lactococcus Lactis, Lactobacillus kefiranofaciens, Bifidobacterium breve* and *Limosilactobacillus reuteri*	In vivo murine model	Daily administration by oral gavage for 3 weeks	Increased capacity of GABA production in the gut microbiota of mouse	[38]
Unpasteurized milk and dairy products	*Lactobacillus*	Clinical trial	Free consumption before and after 12 weeks	Decreased stress and anxiety scores in adults	[39]
Yogurt	*Lactobacillus gasseri* SBT2055 (5×10^8 CFU/dose) and	Clinical trial	100 g/day for 12 weeks	Levels of the stress-induced hormone adrenocorticotrophic hormone significantly decreased in adults	[40]

Finally, the physical handling of animals is also important, because it can interfere in the physiology and behavior of subjects. It is advisable that this handling occurs daily for the animals to adapt to human physical contact [43]. Moreover, the way in which this handling is carried out is important. Mice respond better to passive handling, such as a tube or cupped hands, than to the more usual method, that is the rapid handling by grasping the base of the tail [44].

4 Materials

4.1 Probiotic Strain (s)

As mentioned before, mono- or multi-species microbial formulations can be chosen (*see* **Note 1**). Probiotic cultures can be acquired in lyophilized form for direct addition (Direct Vat Set – DVS) widely used on an industrial scale for its practicality or can be isolated from fermented foods and dairy products.

4.2 Isolation of Probiotic Strains

To perform the isolation of probiotic strains, the following materials are needed:

1. Culturing tools – MRS broth (De Man Rogosa and Sharpe) and MRS agar.
2. Distilled water.
3. 0.1% (w/v, pH 7) peptone water (*see* **Note 2**).
4. Laboratory tools – Petri dishes (90 mm diameter), spatula, glass pipet, micro-pipet, laboratory glassware, sterile plastic bag, and sterile loop.
5. Laboratory equipment – analytical balance, microwave, autoclave, bacteriological incubator, vortex mixer and sample homogenizer.
6. Chemical reagent – 30% hydrogen peroxide (H_2O_2).
7. Culture media should be prepared according to the manufacturer's instructions provided on the label (*see* **Note 3**). After accurately weighing the dehydrated medium, pour all ingredients in an Erlenmeyer flask or any volumetric flask and dilute to 1 L with distilled water. Then mix until complete dissolution using a glass rod and heat (*see* **Note 4**). In the same way, prepare the 0.1% (w / v, pH 7) peptone water. For 1 L of solution, weigh 1.0 g of bacteriological peptone and dilute with 1000 mL of distilled water. Mix until complete dissolution. It is not necessary to warm up. Afterwards, verify that the pH of the media and the peptone water is adequate and proceed to sterilization in an autoclave at 121 °C for 15 min. After removal from the autoclave, identify them correctly with name and date.

4.3 Behavioral Tests

To perform the behavioral tests, add a method of taking notes and collecting data. For this purpose, stopwatches and counters are important to quantify the different experimental variables. This data collection can be done in real time, as long as the researchers are attentive and experienced. However, it is more advisable that the tests are recorded by some camera recording system and analyzed later. Some computerized video tracking system (e.g., Noldus Ethovision, Anymaze) can be used to analyze behavioral tests (*see* **Note 5**). Although these software are helpful, they are very costly and not essential. Whether the experimenter chooses pen and paper or a computer-based entry system depends on budget and convenience.

The apparatus used to carry out the behavioral tests can be purchased or made by the researchers themselves, in compliance with the specifications previously described in the protocol. In general, the apparatus needs to be waterproof and easy to clean, such as acrylic or sealed wood. Between each test, it is essential to clean the apparatus with an ethanol solution, since the odor of feces and urine from the previous animals can interfere with the behavior of the following animals. Between each test, it is essential to clean the apparatus with an ethanol solution to remove the odor of feces and urine.

The specifications of the apparatus used to perform the behavioral tests most used for preclinical trials are listed below. We will use the specifications for tests used in mice, as they are the most used animal model for evaluating the psychobiotics effectiveness.

1. Open field test: The apparatus consists of a circular acrylic box (30 cm in diameter) divided into 12 quadrants distributed in two different zones (central and peripheral). Some laboratories also use white acrylic or wood cage (30 cm × 30 cm × 15 cm) divided into 9 or more quadrants (Fig. 1a).
2. Light-dark box test: acrylic or wood cage (45 cm × 27 cm × 27 cm) unequally divided into two chambers by a black partition containing a small opening (Fig. 1b).
3. Elevated plus maze test: the apparatus consisting of four arms (30 cm × 5 cm) were placed 50 cm above the ground. Two opposite arms were delimited by acrylic vertical walls, whereas the other two opposite arms had unprotected edges (open arms) (Fig. 1c).
4. Marble burying test: in this protocol, 20 or 25 glass marbles were evenly spaced in the plastic cage (35 cm × 50 cm × 35 cm) in the presence of the mouse.
5. Tail suspension test: an apparatus used in which the mice should be suspended 60 cm above the adhesive tape placed approximately 1 cm from the tip of the tail.

Fig. 1 Behavioral assays used to measure anxiety-like behavior in rodents: (**a**) Open field test, (**b**) Light-dark box test, and (**c**) Elevated plus maze test

6. Forced swim test: the apparatus consisted of water-filled polypropylene or glass cylinder (radius = 30 cm; depth = 50 cm) at 25 °C. The animals are unable to escape or touch the bottom of the vessel.
7. Sucrose preference test: For the task, mice are presented with two bearing sipper tubes. One tube contains tap water, whereas the second contains a 2–4% sucrose solution. Prior to beginning testing, mice are habituated to the presence of two drinking bottles (one containing 2% sucrose and the other water) in their home cage.

For conducting clinical trials to assess the efficacy of psychobiotics, a simple and quick protocol is the Hospital Anxiety and Depression Scale (HADS). To perform this test, patients must answer a simple questionnaire. Very few people have difficulty completing it, on paper or electronically.

It is important to highlight that although it is not the focus of this chapter, there are very elegant methods, in addition to behavioral tests, to prove the psychobiotics efficacy in the preclinical and clinical trials, such as serum glucocorticoid measurement, quantification of neurotransmitter and cytokines, as well as brain image studies.

5 Methods

5.1 Isolation of Probiotic Strains

1. Weigh 25 g of the sample and transfer it quantitatively to a sterile plastic bag, add 250 mL of 0.1% (w/v, pH 7) peptone water and place in the sample homogenizer for 1 min at 300 rpm (*see* **Note 6**).
2. Make tenfold dilutions by adding the 1 mL aliquot of the sample to 9 mL of 1% (w/v, pH 7) sterile peptone water, and homogenize each dilution using a vortex mixer. Transfer 1 mL aliquot of each selected dilution to Petri dishes and add 12–15 mL of MRS agar (pour plate technique) (*see* **Note 7**). Subsequently, incubate the plates under anaerobic conditions at 37 °C for 24–48 h (*see* **Note 8**).

3. Select colonies with a circular shape, creamy texture, and white color to be cultivated in MRS broth for 24 h under anaerobic conditions at 37 °C (Fig. 1). After the incubation period, seed again on plates containing MRS agar until complete isolation is achieved.
4. The isolated colonies must be analyzed in relation to their morphological characteristics by the Gram stain method. Gram-positive colonies should proceed for the Catalase test.

   Catalase test.

   Procedure – using a sterile loop, add colony to a glass slide and add 2 drops of 30% (v/v) hydrogen peroxide (H_2O_2).

   Data interpretation: the formation of gas bubbles indicates the production of catalase enzyme by the test bacterium, while no bubble formation is negative for catalase.
5. Purified Gram positive and catalase negative strains are considered presumptive lactic acid bacteria. They can be kept as frozen stocks in 20% (v/v) glycerol at −20 °C until time of use [45].

5.2 Psychobiotic Food Processing

As mentioned earlier, in functional fermented foods, probiotic microorganisms with psychobiotic properties can be part of the raw material whose fermentation process occurs naturally or intentionally through the incorporation of probiotic cultures into the food matrix.

In the production of Kefir, for example, fermentation can occur naturally with lactic acid cultures made with Kefir grains, *Lentilactobacillus kefir*, species of the *Leuconostoc*, *Lactococcus*, and *Acetobacter genera*, producing lactic acid, ethanol, and carbon dioxide [46]. The microbiota of grains is composed of lactose-fermenting yeasts (*Kluyveromyces marxianus*), non-lactose fermenting yeasts (*Saccharomyces omnisporus*, *Saccharomyces cerevisiae* and *Saccharomyces exiguus*), *Streptococcus salivarius* subsp. *thermophilus*, and different probiotic bacteria of the genus *Bifidobaterium* spp., *Lacticaseibacillus casei, L. heveticus, Lacticaseibacillus rhamnosus,* among others, which vary according to the origin and culture method applied [47]. It is important to emphasize that the proof of the beneficial effect is, as a rule, strain-specific and as kefir grains of different origins contain mixtures of different species of microorganisms, for evidentiary purposes, studies in humans must be carried out with the same mixture in which the alleged effect is intended to be demonstrated.

The intentional addition of probiotic cultures to foods as functional ingredients can occur at different stages of processing:

1. As a starter culture itself. Example: fermented vegetables. In the process of making fermented soybeans, the inoculated probiotic strain is exclusively responsible for the fermentation of soybeans at 37 °C for 24 h [35].

2. After the fermentation phases. During the processing of probiotic yogurt, the probiotic culture can be added after fermentation to the cooled (41 °C) product before packaging, simultaneously with the conventional yogurt cultures (*S. thermophilus* and *Lactobacillus delbrueckii bulgaricus*), as well, they can be incorporated as live probiotic starter cultures [48].

5.3 Assessment of Probiotic Cell Viability

Probiotics can lose their viability and metabolic activity due to unfavorable environmental conditions during the production stages and storage period of food products (*see* **Note 9**). In general, the minimum concentration for a given health benefit should be equal to or greater than 6 log CFU/g/mL of probiotic bacteria at the time of consumption [49], which is in compliance with the results reported in Table 2. Therefore, it is necessary to perform the total count of probiotics during the commercial validity of the product, according to the protocol described below.

1. Make ten-fold dilutions by adding the 1 mL aliquot of the sample to 9 mL of 1% (w/v, pH 7) sterile peptone water, homogenize each dilution using a vortex mixer and sowing on plates containing MRS agar by the pour plate technique. Subsequently, incubate the plates under anaerobic conditions at 37 °C for 72 h.
2. Microbial counts are expressed as log CFU/mL.
3. Analyze the morphological characteristics of the colonies by the Gram staining method and catalase test.

5.4 Behavioral Tests

5.4.1 Open Field Test

1. Procedure: Each mouse is placed individually in the center of apparatus and allowed to explore the cage for 5 min (*see* **Note 10).**
2. Variables measured: During this time, the number of squares crossed, number of rearing (standing on hind legs with paws pressed against the wall of the arena), time of grooming, time in the center zone, center distance (the distance traveled in the center of the arena), and center ratio (center distance to total distance ratio) were assessed. At the end of testing, the number of fecal pellets was also counted, and the arena was cleaned with a 10% ethanol solution.
3. Data interpretation: This test is a simple protocol, whose evaluation of its components has been widely used to determine emotionality [50, 51] and spontaneous locomotor activity in rodents [52]. In addition, this test is also considered an excellent model sensitive to drugs with anxiolytic properties. In this test, locomotor activity is indicated by the total distance traveled in the apparatus, while the vertical activity is assigned by the number of rearing. Concerning defecation, this

parameter appeared, under some circumstances, to represent an emotional behavior. Lastly, anxiety-like responses were linked to time in the center zone and center ratio, whereas grooming time indicates higher stress responsiveness.

5.4.2 Light-Dark Box Test

1. Procedure: The animals are individually placed in the test. Mice are placed inside the dark side and allowed to freely move between the two chambers for 5 min (*see* **Note 11**).
2. Variables measured: During this time, the time spent on the light side, number of transitions, and latency to first entry into the light side were recorded.
3. Data interpretation: This test is based on the rodents' innate aversion to brightly lit places and novel environments [53]. In this test, the latency to first entry into the light side and the time spent on the light side, number of transitions, and are associated with anxiety-like behavior. At first, the number of transitions is associated with spontaneous locomotor activity. However, when analyzed with the other variables, it may indicate anxiogenesis.

5.4.3 Elevated Plus Maze Test

1. Procedure: The animals are placed in the center of the maze and allowed to move freely for 5 min. An arm entry was defined as the entry of four paws into the arm (*see* **Note 12**).
2. Variables measured: During this period, the cumulative time and frequency of entries into open and closed arms were registered. Then, the percentage of entries and time in open arms and the time in the central platform were calculated. During the test, the number of stretch-attend posture (SAP), which is generally associated with anxiety, occurs when the rodent elongates its body, and is either standing still or moving forward very slowly.
3. Data interpretation: The elevated plus maze reflects a conflict between the rodents' preference for protected areas and their natural motivation to explore new environments [54]. Thus, this test is used to assess anxiety-like behavior [55]. The anxiety-like behavior is linked to the percentage of entries and time in open arms. In mice, the SAP behavior occurs when the mouse is undergoing risk-assessment specifically due to an internal exploratory-anxiety conflict [56].

5.4.4 Marble Burying Test

1. Procedure: In this protocol, 25 glass marbles were evenly spaced in the presence of the mouse. After 30 min, marbles that were up to two-thirds covered were counted (*see* **Note 13**).
2. Variables measured: The number of marbles buried is evaluated during the test.

3. Data interpretation: In both natural and laboratory conditions, rodents spontaneously use available bedding material to bury unpleasant sources of discomfort present in their home environment. The number of marbles buried is directly related to anxiety response.

5.4.5 Tail Suspension Test

1. Procedure: Mice are suspended by the tail with adhesive tape attached ~1 cm from the tip for 6 min.
2. Variables measured: During the test, the time of immobility and latency to the first immobility episode are evaluated.
3. Data interpretation: This test is based on the observation that rodents, after the initial execution of oriented escape movements, develop an immobile posture when placed in an unavoidable stressful situation [57]. In this test, the condition involves inescapable hemodynamic stress caused by the animals being suspended by the tail. The immobility assumes a low resilience, and consequently, a high level of depression-like behavior (*see* **Note 14**).

5.4.6 Forced Swim Test

1. Procedure: The mice are introduced individually into the water-filled cylinder.
2. Variables measured: During the test, the time of immobility and latency to the first immobility episode are evaluated (*see* **Note 15**).
3. Data interpretation: The forced swim test is also based on the development of an immobile posture immediately following a stressful situation. It is known that immobility time is indicative of low resilience and highly associated with depression-like behavior [58].

5.5 Sucrose Preference Test

1. Procedure: The mice should be placed alone in their home cages in order to accurately measure sucrose solution intake. After 24 or 48 h of acclimation, the mice have the free choice of either drinking the 2% sucrose solution or tap water for a period of 4 days (*see* **Note 16**).
2. Variables measured: Water and sucrose solution intake is measured daily. Sucrose preference is calculated as a percentage of the volume of sucrose intake over the total volume of fluid intake and averaged over the 4 days of testing.
3. Data interpretation: The sucrose preference test for rodents is based on the animal's natural preference for sweets. A low preference for sucrose indicates anhedonia and, consequently, depression-like behavior [59].

5.5.1 Hospital Anxiety and Depression Scale (HADS)

1. Procedure: After patient consent, the researchers should ask them to complete the questionnaire. The researchers should be available to hear doubts or problems related to the participants.
2. Variables measured: The HADS is composed of 14 items, 7 of which is related to the anxiety assessment of (HADS-A) and the other 7 questions are related to depression (HADS-D). Each of your items can be scored from 0 to 3, composing a maximum score of 21 points for each.
3. Data interpretation: The HADS was initially developed to assess psychological distress in non-psychiatric patients. Subjects scores of less than 7 indicate non-cases of significant clinical symptoms for anxiety and/or depression, scores between 8 and 10 indicate mild symptoms, scores between 11 and 14 denote moderate symptoms and scores between 15 and 21 designate severe symptoms of anxiety and/or depression [60].

6 Notes

1. Formulations with multiple strains must have a proven beneficial effect of probiotic mixture. In the case of formulations whose strains have already been recognized for their effectiveness, it is not necessary to carry out a new evaluation [61]. However, it would be ideal to be able to compare the psychotropic properties of each strain present within the multi-strain products versus multi-strain probiotics because the combination can generate synergistic effects on the bioactivity of the probiotics, as well as, mutual inhibition between the strains can occur, reducing the effectiveness of the psychobiotic product [62].
2. 0.1% (w/v, pH 7) peptone water can be replaced with saline solutions, such as (0,85% NaCl, pH 7) sterile saline or phosphate-buffered saline solution (PBS, pH 7).
3. It is recommended to prepare just enough medium for each test. After cooling the MRS agar, distribute approximately 15 mL of the sterile medium in a Petri dish. Then just store the Petri dishes below 5 °C until further use.
4. Be extremely careful when heating to prevent the culture medium to be overheated or burnt.
5. If using a video tracking system, it is necessary that the color of the animal and the apparatus background are different. For lighter animals, we use a dark background. If the animal is darker, we use the white background. Lack of contrast can interfere with the video tracking system performance.

6. All bacteriological procedures need to be performed using a Bunsen burner in a clean and closed working area or inside a sterile laminar flow hood or similar.
7. Before adding MRS agar, make sure it is not too hot. After pouring on the plate, homogenize slowly so as not to spread it on the wall or cover of the plate. Wait for the medium to solidify completely before taking it to a bacteriological oven.
8. 24 h for lactobacilli and 48 h for bifidobacteria.
9. Several factors can contribute to a significant reduction in the viability of probiotic cultures, such as chemical composition of the food matrix, salt and sugar content, type of probiotic strain and its interactions with the starter cultures, inoculation rate, content and availability of nutrients, fermentation temperature and duration, redox potential and dissolved oxygen, pH and titratable acidity, concentration of final metabolites, and storage temperature [22].
10. Generally, the animals are placed in the center of the apparatus to avoid misinterpretations, only counting the central area time after the animal's first spontaneous entry into that zone. Often animals treated with sedative drugs have higher immobility time in the central area. These animals do not show anxiolysis, but rather sedation.
11. In the light-dark box test, a very common mistake is the use of a low brightness environment. In these circumstances, there will not be much contrast and, consequently, the light side will be less aversive. Based on this premise, it is essential to use a luxmeter to measure light intensity. 400-lux illumination in the white area was sufficiently aversive to significantly reduce the time spent in that area [53].
12. It is necessary to standardize the way in which rodents are handled. Make sure that each animal will be placed inside the apparatus in the same position. Differences are observed when rodents are placed facing toward an open arm versus toward the closed arm [54]. In our laboratory, rodents are placed on the maze facing the same closed arm.
13. In the marble burying test, substrates of a denser nature (i.e., river sand) should be used, since this prevents incidental settling of marbles during the test. In addition, video recording of the test is recommended in order to accurately differentiate marble-directed behavior, as opposed to an accidental covering of marbles caused by the normal exploration or by an increased locomotor activity of the animals [63].

14. Any manipulation that increases the general motor activity of the animals is capable of reducing the immobility time, which can lead to spurious conclusions. For this reason, it is essential to evaluate mice locomotor activity previously through the open field test [64].
15. Immobility is defined as floating vertically in water, making only those minimal movements necessary to keep the head above water [65].
16. The positions of the two bottles or tubes in each cage or chamber should be changed each day. In addition, make sure the bottles used in the protocol are not leaking [59].

References

1. James SL, Abate D, Abate KH et al (2018) Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990–2017: a systematic analysis for the global burden of disease study 2017. Lancet 392:1789–1858
2. Cohen BE, Edmondson D, Kronish IM (2015) State of the art review: depression, stress, anxiety, and cardiovascular disease. Am J Hypertens 28:295–1302
3. Tolentino, JC. Schmidt, SL (2018) DSM-5 criteria and depression severity: implications for clinical practice. Psychiatr Front 9: 450
4. NIMH (2022) Anxiety Disorders. Available online: https://www.nimh.nih.gov/health/topics/anxiety-disorders/index.shtml#part_145335. Accessed on 19 July 2022
5. NIMH (2022) Depression. Available online: https://www.nimh.nih.gov/health/topics/depression/index.shtml#part_145399. Accessed on 19 July 2022
6. Zhu H, Tian P, Zhao J, Zhang H, Wang G, Chen W (2022) A psychobiotic approach to the treatment of depression: a systematic review and meta-analysis. J Funct Foods 91: 104999
7. Akkasheh G, Kashani-Poor Z, Tajabadi-Ebrahimi M et al (2016) Clinical and metabolic response to probiotic administration in patients with major depressive disorder: a randomized, double blind, placebo-controlled trial. Nutrition 32:315–320
8. Misra S, Mohanty D (2017) Psychobiotics: a new approach for treating mental illness? Crit Rev Food Sci Nutr:1–7
9. Dinan TG, Stanton C, Cryan JF (2013) Psychobiotics: a novel class of psychotropic. Biol Psychiatry 74:720–726
10. Sarkar A, Lehto SM, Harty S et al (2016) Psychobiotics and the manipulation of bacteria-gut-brain signals. Trends Neurosci 39:763–781
11. Mohammadi AA, Jazayeri S, Khosravi-Darani K et al (2016) The effects of probiotics on mental health and hypothalamic-pituitary-adrenal axis: a randomized, double-blind, placebo-controlled trial in petrochemical workers. Nutr Neurosci 19:387–339
12. Takada M, Nishida K, Kataoka-Kato A et al (2016) Probiotic *lactobacillus casei* strain Shirota relieves stress-associated symptoms by modulating the gut–brain interaction in human and animal models. Neurogastroenterol Motil 28:1027–1036
13. Denayer T, Stöhr T, Van Roy M (2014) Animal models in translational medicine: validation and prediction. New Horiz Transl Med 2:5–11
14. Nestler EJ, Hyman SE (2010) Animal models of neuropsychiatric disorders. Nat Neurosci 13:1161–1169
15. Magalhães-Guedes KT (2022) Psychobiotic therapy: method to reinforce the immune system. Clin Psychopharmacol Neurosci 20:17–25
16. Kadosh KC, Basso M, Knytl P et al (2021) Psychobiotic interventions for anxiety in young people: a systematic review and meta-analysis, with youth consultation. Transl Psychiatry 11:352
17. Cryan JF, O'Riordan KJ, Cowan CSM et al (2019) The microbiota-gut-brain Axis. Fisiol Ver 99:1877–2013
18. Luang-In V, Katisart T, Konsue A et al (2020) Psychobiotic effects of multi-strain probiotics originated from Thai fermented foods in a rat model. Food Sci Anim Resour 40:1014–1032

19. Casertano M, Fogliano V, Ercolini D (2022) Psychobiotics, gut microbiota and fermented foods can help preserving mental health. Food Res Int 152:110892
20. Benton D, Williams C, Brown A (2007) Effects of consumption of a milk drink containing a probiotic on mood and cognitive function. Eur J Clin Nutr 61:355–361
21. Ortega MA, Fraile-Martinez O, Garcia-Montero S et al (2022) Biological role of nutrients, food and dietary patterns in the prevention and clinical management of major depressive disorder. Nutrients 14:103390
22. Khorshidian N, Yousefi M, Mortazavian AM (2020) Fermented milk: the most popular probiotic food carrier. Adv Food Nutr Res 94:91–114
23. Park S, Kang S, Jeong D-Y et al (2016) Black carrots fermented with *lactobacillus plantarum* or aspergillus oryzae prevent cognitive dysfunction by improving hippocampal insulin signalling in amyloid-β infused rats. J Funct Foods 25:354–366
24. Ko CY, Lin H-TV, Tsai GJ (2013) Gamma-aminobutyric acid production in black soybean milk by *lactobacillus brevis* FPA 3709 and the antidepressant effect of the fermented product on a forced swimming rat model. Process Biochem 48:559–568
25. Yu L, Han X, Cen S et al (2020) Beneficial effect of GABA-rich fermented milk on insomnia involving regulation of gut microbiota. Microbiol Res 233:126409
26. Tillisch K, Labus J, Kilpatrick L et al (2013) Consumption of fermented milk product with probiotic modulates brain activity. Gastroenterol 144:1394–1401.e4
27. Chung Y-C, Jin H-M, Cui Y et al (2014) Fermented milk of *lactobacillus helveticus* IDCC3801 improves cognitive functioning during cognitive fatigue tests in healthy older adults. J Funct Foods 10:465–474
28. Akbari E, Asemi Z, Daneshvar Kakhaki R et al (2016) Effect of probiotic supplementation on cognitive function and metabolic status in Alzheimer's disease: a randomized, double-blind and controlled trial. Front Aging Neurosci 2016:8
29. Musa NH, Mani V, Lim SM et al (2017) Lactobacilli-fermented cow's milk attenuated lipopolysaccharide-induced neuroinflammation and memory impairment in vitro and in vivo. J Dairy Res 84:488–495
30. Nishida K, Sawada D, Kawai T et al (2017) Para-psychobiotic *lactobacillus gasseri* CP 2305 ameliorates stress-related symptoms and sleep quality. J Appl Microbiol 123:1561–1570
31. Sawada D, Kawai T, Nishida K et al (2017) Daily intake of lactobacillus gasseri CP2305 improves mental, physical, and sleep quality among Japanese medical students enrolled in a cadaver dissection course. J Funct Foods 31: 188–197
32. Ohsawa K, Nakamura F, Uchida N et al (2018) *Lactobacillus helveticus*-fermented milk containing lactononadecapeptide (NIPPLTQTPVVVPPFLQPE) improves cognitive function in healthy middle-aged adults: a randomised, double-blind, placebo-controlled trial. Int J Food Sci Nutri 69:369–376
33. Reid SNS, Ryu J, Kim Y et al (2018) The effects of fermented Laminaria japonica on short-term working memory and physical fitness in the elderly. Evid Based Complement Alternat Med 2018:8109621
34. Jung Park H, Shim HS, Lee GR et al (2019) A randomized, double-blind, placebo-controlled study on the memory-enhancing effect of lactobacillus fermented Saccharina japonica extract. Eur J Integ Med 28:39–46
35. Hwang YH, Park S, Paik J et al (2019) Efficacy and safety of lactobacillus plantarum C29-fermented soybean (DW2009) in individuals with mild cognitive impairment: a 12-week, multi-center, randomized, double-blind, placebo-controlled clinical trial. Nutrients 11(10):3390
36. Kim KM, Yu KW, Kang DH et al (2002) Anti-stress and anti-fatigue effect of fermented rice bran. Phytother Res 16:700–702
37. Ton AMM, Campagnaro BP, Alves GA et al (2020) Oxidative stress and dementia in Alzheimer's patients: effects of Synbiotic supplementation. Oxidative Med Cell Longev 2020: 2638703
38. Van de Wouw M, Walsh AM, Crispie F et al (2020) Distinct actions of the fermented beverage kefir on host behaviour, immunity and microbiome gut-brain modules in the mouse. Microbiome 8:67
39. Buttler MI, Bastiaanssen TFS, Long Smith C et al (2020) Recipe for a healthy gut: intake of unpasteurised milk is associated with increased lactobacillus abundance in the human gut microbiome. Nutrients 12:1468
40. Nishihira J, Kagami-Katsuyama H, Tanaka A et al (2014) Elevation of natural killer cell activity and alleviation of mental stress by the consumption of yogurt containing *lactobacillus gasseri* SBT2055 and *Bifidobacterium longum* SBT2928 in a double-blind, placebo-controlled clinical trial. J Funct Foods 11:261–268
41. Del Toro-Barbosa M, Hurtado-Romero A, Amezquita LEG et al (2020) Psychobiotics:

mechanisms of action, evaluation methods and effectiveness in applications with food products. Nutrients 12:3896. https://doi.org/10.3390/nu12123896
42. Rodgers RJ, Cole JC (1993) Influence of social isolation, gender, strain and prior novelty on plus-maze behaviour in mice. Physiol Behav 54:729–736
43. Balcombe JP, Barnard ND, Sandusky C (2004) Laboratory routines cause animal stress. Contemp Top Lab Anim Sci 43:42–51
44. Hurst JL, West RS (2010) Taming anxiety in laboratory mice. Nat Methods 7:825–826
45. Sakkaa SE, Zaghloul EH, Ghanem KM (2022) Psychobiotic potential of gamma-aminobutyric acid–producing marine enterococcus faecium SH9 from marine shrimp. Probiotics Antimicro Prot 14:934
46. Brasil (2007) Ministério da Agricultura, Pecuária e Abastecimento (MAPA). Instrução Normativa N° 46, de 23 de outubro de. Regulamento Técnico de Identidade e Qualidade de Leites Fermentados. Brasília – DF. Diário Oficial da República Federativa do Brasil, 24 de outubro de 2007. Seção 1, Página 5
47. Bourrie BCT, Willing BP, Cotter PD (2016) The microbiota and health promoting characteristics of the fermented beverage kefir. Front Microbiol 7:1–17
48. Lourens-Hattingh A, Viljoen BC (2001) Yogurt as probiotic carrier food. Int Dairy J 11:1–17
49. Hill C, Guarner F, Reid G et al (2014) Expert consensus document. The international scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol 11:506–514
50. Roth KA, Katz RJ (1979) Stress, behavioral arousal, and open field activity – a reexamination of emotionality in the rat. Neurosci Biobehav Rev 3:247–263
51. Seibenhener ML, Wooten MC (2015) Use of the open field maze to measure locomotor and anxiety-like behavior in mice. JoVE 96:e52434
52. Walsh RN, Cummins RA (1976) The open-field test: a critical review. Psychol Bull 83:482–504
53. Bourin M, Hascoët M (2003) The mouse light/dark box test. Eur J Pharmacol 463:5–65
54. Pellow S, Chopin P, File S et al (1985) Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 14:149–167
55. Archer J (1973) Tests for emotionality in rats and mice: a review. Anim Behav 21:205–235
56. Mikics É, Barsy B, Barsvári B et al (2005) Behavioral specificity of non-genomic glucocorticoid effects in rats: effects on risk assessment in the elevated plus-maze and the open-field. Horm Behav 48:52–162
57. Porsolt RD, Le Pichon M, Jalfre ML (1977) Depression: a new animal model sensitive to antidepressant treatments. Nature 266:730–732
58. Porsolt RD, Bertin A, Blavet N et al (1979) Immobility induced by forced swimming in rats: effects of agents which modify central catecholamine and serotonin activity. Eur J Pharmacol 57:201–210
59. Liu MY, Yin CY, Zhu LJ et al (2018) Sucrose preference test for measurement of stress-induced anhedonia in mice. Nat Protoc 13:1686–1698
60. Zigmond AS, Snaith RP (1983) The hospital anxiety and depression scale. Acta Psychiatr Scand 67:361–370
61. Anvisa (2022) Agencia Nacional de Vigilância Sanitária. Brazillian Health Surveillance Agency. Guia para Instrução Processual de Petição de Avaliação de Probióticos para Uso em Alimentos. Guia 21/2021 – versão 2. Available online: http://antigo.anvisa.gov.br/documents/10181/5280930/guia+21+v2.pdf/dac5bf5f-ae56-4444-b53c-2cf0f7c15301. Accessed on 19 Sep 2022
62. Chapman CM, Gibson GR, Rowland I (2011) Health benefits of probiotics: are mixtures more effective than single strains? Eur J Nutr 50:1–17
63. De Brouwer G, Fick A, Harvey BH et al (2019) A critical inquiry into marble-burying as a preclinical screening paradigm of relevance for anxiety and obsessive–compulsive disorder: mapping the way forward. Cogn Affect Behav Neurosci 19:1–39
64. Can A, Dao DT, Terrillion CE et al (2012) The tail suspension test. JoVE 59:e3769
65. Abel EL (1993) Ontogeny of immobility and response to alarm substance in the forced swim test. Physiol Behav 54:713–716

Correction to: Paraprobiotics Preparation for Use in Food and Beverages

Cássia Pereira Barros, Roberto Pessanha da Silva Pires, and Jonas de Toledo Guimarães

Correction to:
Chapter 15 in: Adriano Gomes da Cruz et al. (eds.), *Probiotic Foods and Beverages: Technologies and Protocols*, Methods and Protocols in Food Science,
https://doi.org/10.1007/978-1-0716-3187-4_15

The original version of Chapter 15 had incorrect author group listed: Cássia Pereira Barros, Cynthia Manassi, Silvani Verruck, Marcia Cristina Silva, Erick A. Esmerino, Monica Q. Freitas, and Adriano Gomes da Cruz. This has now been rectified.

The correct author group is **Cássia Pereira Barros, Roberto Pessanha da Silva Pires, and Jonas de Toledo Guimarães**, and the corresponding author is **Cássia Pereira Barros**.

The updated original version of this chapter can be found at
https://doi.org/10.1007/978-1-0716-3187-4_15

Adriano Gomes da Cruz et al. (eds.), *Probiotic Foods and Beverages: Technologies and Protocols*,
Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-3187-4_18,
© The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2023

Index

A

Acetic acid 48, 69, 89, 133, 134, 136, 140, 156, 194, 224, 225, 239
Acid coagulation 38, 39
Acidifying 35, 94, 156
Additives 7, 9, 13, 15, 17, 18, 20, 24, 62, 101, 106, 160, 193, 238
Allergenicity 81
Almond 24, 94
Antagonistic effect 15, 214
Antibiotics 9, 124, 236
Antimicrobial components 15
Anxiety 214, 243, 244, 247, 248, 250, 253, 256–258
Aromatic compounds 69, 143, 160, 175

B

Bacteriocins 27, 69, 106, 121, 224, 228, 236, 237, 239
Bakery 55, 68, 86, 165–168, 175, 232
Beer 139–149
Behavior test 247–251
Bifidobacterium 3, 20, 26, 36, 42, 46, 53, 57–59, 72, 73, 83, 95, 105, 110, 113, 121, 140, 141, 153, 155, 166, 167, 171, 172, 180, 184, 186, 194, 200, 220, 234, 245, 246, 248–250
Bioactivities 69, 239, 258
Biofilms 225, 236
Biological activity 214, 246
Biosurfactants 224
Blending 95, 180, 181
Bovine breeds 68
Bread 166–170, 172–176
Buffering capacity 36, 247
Bulking agents 180, 182, 183
Butter 61, 68–78, 179, 181, 182, 187, 189

C

Canestrato Pugliese cheese 44
Carrier 36, 37, 105, 120, 201, 247
Cashew nuts 94
Cattle breeds 7
Cell cultivation 56
Cell-free supernatant (CFS) 224–232, 234, 235, 239
Cell inactivation 5
Cheddar cheese 37, 44, 46, 47, 49
Chocolates 61, 68, 179–194
Churning 69–71, 73–77
Chymosin 38, 40–42
Coagulant enzymes 35
Coconut oil 94
Color measurement 191
Conching 180, 182, 187, 189, 193
Conjugated linoleic acid (CLA) 45, 68, 69, 75, 156
Contaminants 8, 9, 16–18, 28
Cooling 9, 13, 17, 41, 55, 71, 75–77, 84, 88–90, 107, 123, 157, 176, 180, 189, 218, 219, 239, 258
Corn zein 94
Cottage cheese 40
Cream maturation 70, 76
Crescenza 41
Critical control point (CCP) 14, 16, 18, 23
Curd 17, 28, 35–37, 39–44, 47, 97–99

D

Dark chocolate 180, 181, 189, 193
Deaeration 9
Demineralization 35
Depression 243, 244, 250, 253, 258
Determination of catechins ..... vii, 136–137
Determination of sugars ..... vii, 136
Direct vat 40, 56, 62, 73, 83, 129, 159, 169, 171, 215, 218, 227, 251

E

Edible films 166, 176
Elevated plus maze test 256
Emulsion 47, 61, 68, 71, 82, 95, 96, 100, 199–203, 206, 207
Encapsulation 8, 24, 45, 63, 108, 109, 111, 114, 141, 166, 172, 175, 200–205, 207, 209
Enzymatic hydrolysis 37

Adriano Gomes de Cruz et al. (eds.), *Probiotic Foods and Beverages: Technologies and Protocols*, Methods and Protocols in Food Science, https://doi.org/10.1007/978-1-0716-3187-4,

© The Editor(s) (if applicable) and The Author(s), under exclusive license to Springer Science+Business Media, LLC, part of Springer Nature 2023

Equipment 8, 9, 48, 55, 60, 70, 74, 76, 77, 139, 144, 145, 148, 159, 167, 182, 187, 189, 205, 206, 216, 228, 251
Ethanol 6, 9, 69, 139–143, 239, 252, 254, 255
Exopolysaccharides 11, 69, 121, 233
Extrusion 8, 47, 72, 109, 199–204, 206, 207

F

Feeding material 8
Fermentation 2, 8, 11, 13–17, 19, 21, 23, 24, 37, 40, 47, 62, 68, 69, 71, 73, 81–84, 86–89, 94–97, 100–102, 106, 107, 120, 121, 125–130, 133, 135, 140, 142–148, 151, 153, 155, 161, 169, 172–174, 224, 226–228, 234, 235, 246, 254, 255, 259
Fermented cream 40, 68, 75
Fermented milk 1, 2, 5–9, 11, 13–28, 36, 62, 105, 244, 247, 248, 250
Fermented vegetables 120–125, 254
Festivo cheese 42
Feta cheese 43, 46
Filtration 9, 14, 84–87, 97, 100, 101, 147, 148, 203, 204, 226, 229–231, 234
Flavor 8, 15, 17, 18, 23, 27, 28, 35, 38, 39, 44, 48, 61, 62, 69, 82–84, 89, 93, 94, 98, 106, 107, 137, 140, 147, 148, 152, 156, 160, 172, 173
Forced swim test 257
Free fatty acids (FFAs) 45
Freeze-drying 200, 231
Fresh cheese 36, 40, 41, 46
Frozen desserts 53
Fruits 6, 13, 14, 16, 18, 26, 55, 88, 89, 101, 102, 106–108, 125, 148, 232
Functional products 105, 214
Fusion properties 191

G

Gelation 37, 39, 72, 108, 109, 202, 203

H

Hard cheese 38, 43–46, 48, 49
Hardening 56, 60, 63
Harvesting 229
Homogenization 56, 60, 61, 82, 94, 96, 100, 107, 172, 203
Hospital 253, 258

I

Ice creams 53–58, 60–63
Inactivation processes 215, 218, 220
Inoculation 13–15, 37, 40, 42, 53, 56–58, 71, 72, 83, 87, 88, 96, 111, 123, 129, 137, 156, 159, 169, 171, 180–184, 189, 190, 194, 227–229, 238, 259
Inulin 13, 20, 24, 25, 54, 59, 109, 180, 183, 192, 200

J

Juice 36, 89, 105–109, 111, 112, 114, 115, 121, 124, 125, 137

K

Kombucha 133–135, 137, 246

L

Laboratory scale 44, 48, 129, 144, 148
Lactation season 8
Lactic acid 2, 8, 11, 13, 15, 21, 25, 27, 35, 39, 40, 62, 69, 81, 94, 106, 122, 126–128, 133, 134, 140, 142, 155, 160, 218, 224, 225, 227, 239, 245, 254
Lacticaseibacillus 3, 19–22, 25, 36, 37, 40, 46, 47, 54, 57–59, 72, 73, 83, 95, 112, 125, 126, 140, 143, 158, 159, 166, 167, 171, 172, 180, 184, 194, 200, 201, 245, 246, 249, 254
Lactobacillus 3, 6, 8, 15, 18–21, 25, 36, 46, 47, 53, 57–59, 69, 72, 73, 83, 95, 105–107, 110, 113, 121, 123–126, 140, 142, 149, 155, 166, 167, 171, 172, 180, 184, 194, 200, 234, 245–250, 255
Lactose 8, 27, 35, 53, 61, 68, 82, 105, 165, 186, 190, 194, 247
Lactose intolerance 36, 81, 93, 120, 152
Large-scale production 44, 214
Light-dark box test 253, 256, 259
Limosilactobacillus 4, 41, 46, 83, 180, 184, 194, 200, 236, 245, 246, 249, 250
Live microorganisms 1, 2, 5, 23, 27, 36, 45, 69, 105, 140, 152, 165, 180, 213

M

Marble burying test 259
Maturation 36, 39, 45, 56, 60, 62, 70, 75, 76, 126, 145, 147–149, 154, 159, 162
Mental disorders 243, 244
Microbial propagation 226
Microbial strains 3, 226–228, 233, 245
Microencapsulated 8, 54, 74, 152
Milk chocolate 181, 189
Minas frescal cheese 40
Mold 41, 42, 44, 96, 98, 107, 161, 162, 189
Molding 39, 180, 182, 189
Mucilage 24, 130, 166–168, 170, 175, 200

N

Neurotransmitters 122, 224, 253

O

Oat extract 85, 86
Oil 61, 68, 77, 85, 94–96, 99, 110, 140, 144, 147, 149, 191, 201, 203, 205

P

Packaging..5, 7, 13–15, 17–19, 21–23, 27, 56, 76, 89, 90, 99, 101, 107, 125–127, 147, 176, 182, 189, 193, 194, 203, 204, 207, 225, 233, 255
Palm fruit oil 94
Paracaseinate... 39
Paraprobiotics.............69, 214, 215, 218–220, 223, 224
Particle size distribution 187, 191
Peas 94–97
Penicillium camemberti 39
Physical analysis 190
Pickles 122, 123, 126, 127
Plant-based beverages 81–83, 88
Plant-based cheese 93–95
Prebiotics 13, 20, 24–27, 54, 82, 86, 107, 120, 181, 192, 200, 201, 213, 223, 224, 244
Pre-pasteurization 9
Probiotics... 1–5, 7–9, 11, 13, 15, 16, 18–28, 35–37, 40–49, 53–60, 62, 63, 68, 69, 71–76, 81–83, 86–90, 93–102, 105–114, 119–129, 140–145, 147, 148, 152–160, 162, 165–172, 174–176, 180–184, 186–190, 192–194, 199–210, 213–216, 218–220, 223, 224, 227–229, 232, 234, 244, 245, 247, 251, 253–255, 258, 259
Propagation method 56
Proximate composition... 5
Psychobiotics 244–248, 250, 252–254, 258
Purification 9, 12, 56, 217

R

Raw milk 7–9, 14, 23, 28, 77, 98
Refining 180, 187, 189
Rennet 40, 41, 43–45, 94
Rheological measurements 191–192
Rice extracts...82, 83, 86–88

S

Salt resistance 47
Sanitation 8, 9
Sausages 151–156, 158–160, 215
Semi-hard cheese 44
Semi-soft cheese 42
Sensory acceptance 15
Sensory properties..17, 26, 48, 86, 107, 108, 126, 153, 156
Separation procedures.. 232, 237
Shelf life 36, 42, 54, 69, 95, 107, 108, 113, 120, 142, 152, 172, 190, 233
Sodium caseinate 24, 166–168, 170, 175
Soft cheese 40–42
Soybeans 81, 82, 84, 85, 94, 97, 120–122, 124, 128–130, 201, 203, 248, 249, 254
Soy extract 81, 84, 85
Spontaneous ferments.. 83
Spray dryer..203–205, 210, 226
Stabilizers 20, 55, 56, 59, 61, 94, 100
Standards 2, 6, 9, 20, 43, 68, 113, 226, 238
Starch 13, 20, 25, 44, 82, 86, 87, 94, 97, 171, 173, 175, 201
Starter cultures 7–9, 11, 12, 14–17, 20, 25–28, 35–37, 40, 42, 44, 83, 89, 95–97, 126–128, 134, 137, 140, 142, 151–155, 159, 160, 215, 228, 245, 254, 255, 259
Storage 5, 7, 9, 12–14, 18, 19, 21, 23, 25–27, 36, 40–45, 47, 53, 54, 58, 60, 63, 71, 72, 76, 78, 83, 100, 102, 106–109, 112, 115, 121, 126, 137, 141–143, 147, 153, 155, 157, 162, 166, 171, 180, 182–184, 189, 190, 193, 194, 201, 205, 207–209, 214, 231, 234, 255, 259
Streptococcus...3, 6, 8, 15, 20, 26, 39, 41, 95, 125, 140, 180, 185, 194, 248, 254
Sucrose preference test 257–258
Sugar replacements 181
Suspensions 72, 78, 87, 96, 98, 110, 172, 204, 218–220, 228, 238
Sweet cream...68, 75, 76
Sweeteners 7, 13, 14, 18, 20, 25–27, 55, 61, 106, 180, 182, 192
Symbiotic culture of bacteria and yeast (SCOBY)..133–135, 137
Synbiotics........................ 13, 53, 54, 181–183, 188, 192
Syneresis...25, 35, 39, 100
Synergistic...................................8, 11, 15, 25, 181, 258

T

Tail suspension test 257
Tempering 180, 189
Texture analysis 191
Texturization 43
Thermiziation.. 43
Thickeners 13–15, 18, 20, 24, 25, 88, 89
Tindalization 215

V

Veganism 81
Vegetable proteins 94
Viability 2, 8, 11, 13, 19, 24–26, 36–38, 40–42, 44, 45, 47–49, 53, 54, 63, 69, 71, 90, 100, 102, 106–109, 111, 112, 114, 115, 121, 126, 129, 142, 147, 154, 162, 165, 166, 171, 172, 174–176, 180, 182–184, 190, 193, 194, 199, 208–210, 213, 214, 238, 255, 259
Viable cell count 15, 152, 203–205

W

Washing 87, 125, 127
Water activity 38, 106, 152, 162, 172, 173, 176, 180, 187, 190, 199, 208
White cheese 42, 43
White chocolate 180–182, 189, 193