Chapter 13

Probiotic and Synbiotic Chocolate

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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,

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;

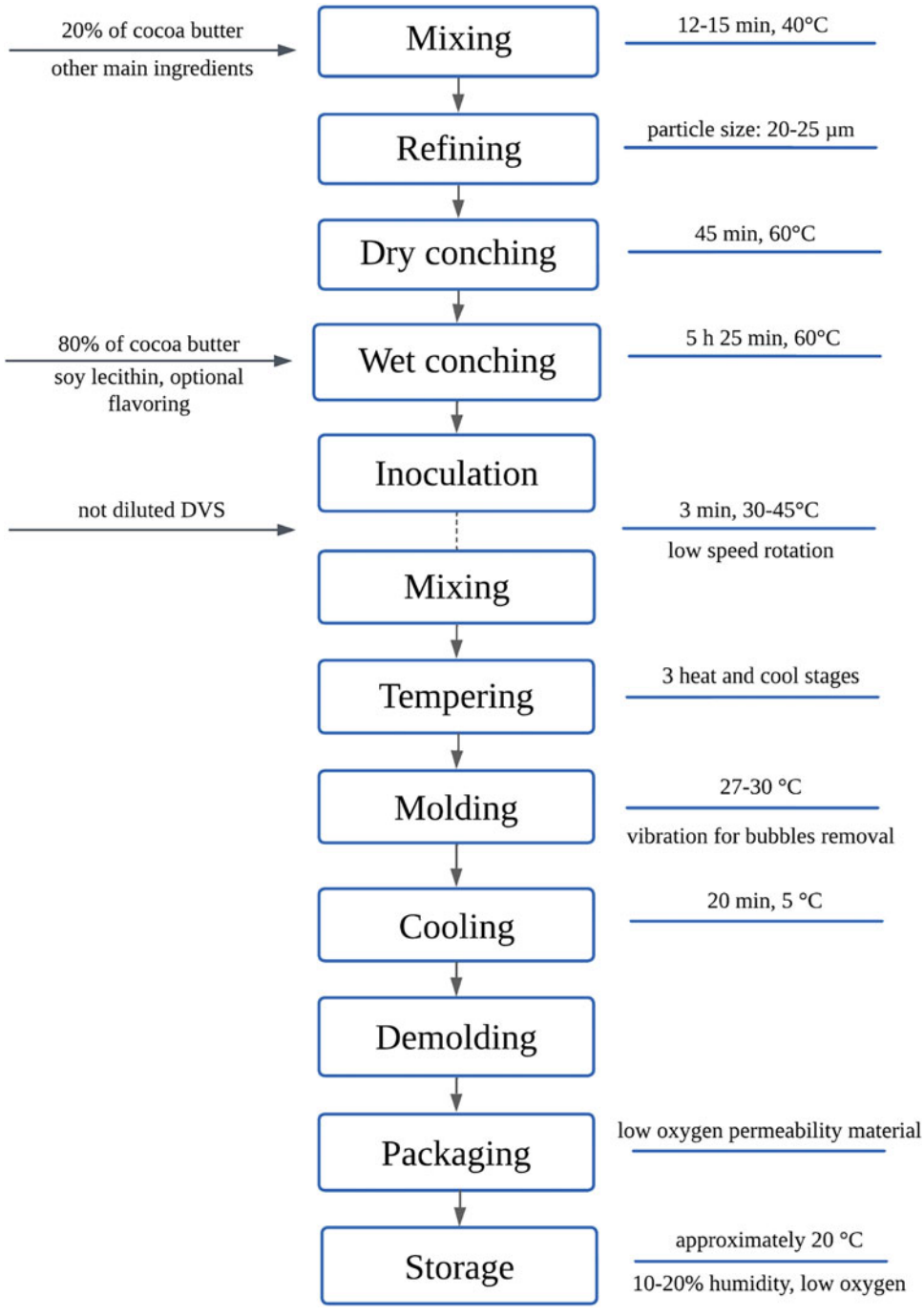

Fig. 1 Flowchart of probiotic and synbiotic chocolate production

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].

$$C^* = \sqrt{a^2 + b^2} \tag{1}$$

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

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].

$$\sqrt{\tau} = \sqrt{\tau_{CA}} + \sqrt{\mu * \mathrm{CA}} * \sqrt{\gamma} \tag{3}$$

Where, τ: yield stress; τ_{CA}: Casson yield stress; $\mu * \mathrm{CA}$: 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