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INCORPORATION OF *BIFIDOBACTERIUM ANIMALIS* SUBSPECIES *LACTIS* BB-12® AND *AKKERMANSIA MUCINIPHILA* IN CHOCOLATE MATRICES

By

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Thesis presented to Escola Superior de Biotecnologia of the Universidade Católica Portuguesa to fulfill the requirements of Master of Science degree in Applied Microbiology

by
Rita da Costa Vedor

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Este trabalho é dedicado aos meus pais - pelo apoio, carinho e amor incondicional.

"The reward of the young scientist is the emotional thrill of being the first person in the history of the world to see something or to understand something. Nothing can compare with that experience."
Cecilia Payne-Gaposchkin

"Trust the process and it will bring out the hidden subject as the results."
David Harris.

Resumo

Atualmente, os consumidores procuram alternativas mais saudáveis para a sua alimentação, priorizando assim alimentos com melhores propriedades organoléticas e com maiores benefícios a nível da saúde. Consequentemente, a indústria alimentar teve de se adaptar a esta procura, concentrando-se assim no desenvolvimento de alimentos funcionais. Estes produtos alimentares caracterizam-se pela incorporação de ingredientes bioativos, que complementam a nutrição essencial (suplemento alimentar) ou que apresentam uma atividade farmacológica (nutracêutica). Os probióticos, definidos como "microrganismos vivos que, quando administrados em quantidades adequadas, conferem um benefício para a saúde humana", são um bom exemplo. Estes têm sido incorporados em diferentes tipologias de matrizes alimentares particularmente matrizes lácteas, e mais recentemente em matrizes cerealíferas ou hortofrutícolas. Numa abordagem de aumentar o portefólio de vetores alimentares atrativos para o fornecimento de bactérias probióticas ao consumidor, foi selecionado e estudado o chocolate por ser um produto com extrema popularidade entre os consumidores. Assim, o objetivo principal da presente tese foi estudar a incorporação de duas estirpes probióticas, nomeadamente *Bifidobacterium animalis* subsp. *lactis* BB-12® (estirpe probiótica clássica) e *Akkermansia muciniphila* DSM 22959 (estirpe candidata a nova geração de probióticos) em três matrizes de chocolate com diferentes percentagens de cacau (33.6%; 54.5% e 70.5%) e avaliar a sua viabilidade e estabilidade ao longo de 28 dias de armazenamento aeróbico e na simulação da passagem do trato gastrointestinal (TGI). Simultaneamente foram quantificados o teor de compostos fenólicos totais e determinadas as seguintes atividades biológicas: antioxidante, antidiabética e anti-hipertensiva.

Para o probiótico *B. animalis* BB-12®, a matriz mais adequada foi o chocolate contendo 70.5% de cacau, uma vez que assegurou níveis de concentração celular de 10^8 UFC/g, após 28 dias de armazenamento aeróbico e assegurou níveis de 10^7 UFC/g durante a simulação *in vitro* do trato gastrointestinal. Além disso, os chocolates com 70.5% de cacau exibiram maior conteúdo de compostos fenólicos totais e bioatividades superiores (antioxidante, antidiabética e anti-hipertensiva). Relativamente a *A. muciniphila* DSM 22959, a matriz de chocolate mais apropriada foi a de 54.5% de cacau, uma vez que a viabilidade celular foi mantida a 10^6 UFC/g, após 28 dias de armazenamento aeróbio. Apesar de *A. muciniphila* ter sobrevivido durante o armazenamento na matriz de chocolate, o probiótico foi incapaz de suportar as condições do TGI, quando incorporada no chocolate. Relativamente a parâmetros como o aspeto e textura, os resultados indicaram que a adição de probióticos modifica a superfície do chocolate e aumenta simultaneamente a dureza destes. Adicionalmente, os resultados de uma prova de análise sensorial (prova triangular) com chocolate com 70.5% de cacau incorporados com *A. muciniphila* demonstraram como mais de 50% dos provadores não consegue distinguir entre um chocolate sem bactéria e o chocolate incorporado com probiótico. Embora a adição de probióticos leve a alterações nas propriedades organoléticas da matriz do chocolate, os benefícios suplementares de um chocolate probiótico compensam estas diferenças, sendo por consequente o chocolate funcional mais atrativo para o consumidor. Em conclusão, uma matriz de chocolate com elevado teor de cacau (chocolate preto) é um método adequado para a incorporação dos probióticos *Bifidobacterium animalis* subsp. *lactis* BB-12® e *Akkermansia muciniphila*.

Palavras-chave: Chocolate**;** *Bifidobacterium animalis* subsp. *lactis* BB-12®; *Akkermansia muciniphila*; Bioatividades; Viabilidade

Abstract

Currently, consumers are seeking healthier food options, prioritizing goods with improved organoleptic and health properties. Consequently, the food industry has been focusing on developing functional foods, which are characterized by the incorporation of bioactive ingredients, which complement essential nutrition (food supplement) or have a pharmacological activity (nutraceutical). Probiotics, defined as "live microorganisms that, when administered in adequate amounts, confer benefits to human health", are a good example. These have been incorporated into different types of food matrices, particularly dairy matrices, and more recently into cereal or fruit and vegetable matrices. In an attempt to increase the portfolio of attractive food vectors for the delivery of probiotic bacteria to the consumer, chocolate was selected and studied due to its extreme popularity among consumers. Thus, the main objective of this thesis was to study the incorporation of two probiotic strains, namely *Bifidobacterium animalis* subsp. *lactis* BB-12® (classical probiotic strain) and *Akkermansia muciniphila* DSM 22959 (candidate strain for new probiotic generation) in three chocolate matrices with different cocoa percentages (33. 6%; 54.5% and 70.5%) and evaluate their viability and stability over 28 days of aerobic storage and in simulated gastrointestinal tract (GIT) passage. Simultaneously, the content of total phenolic compounds was quantified, and the following biological activities were determined: antioxidant, antidiabetic, and antihypertensive.

For the probiotic *B. animalis* BB-12®, the most suitable matrix was the chocolate containing 70.5% cocoa content, as it assured cell concentration levels of 10^8 CFU/g after 28 days of aerobic storage and presented viable cell numbers of 10^7 CFU/g during *in vitro* GIT simulation. Furthermore, the chocolates with 70.5% cocoa presented greater total phenolic compounds content and higher bioactivities (antioxidant, antidiabetic and antihypertensive). Regarding *A. muciniphila* DSM 22959, the most appropriate chocolate matrix was the one with 54.5% cocoa content, as cell viability was kept at 10^6 CFU/g, after 28-day aerobic storage. Though, *A. muciniphila* survived during aerobic storage in the chocolate matrix, this bacterium was unable to endure the simulated GIT conditions, when incorporated into chocolate. Regarding parameters such as overall quality aspect and texture, results indicated that the addition of probiotics modifies the chocolate's surface and increases its hardness. Moreover, the results of a performed sensory analysis test (triangular test) with 70.5% cocoa content chocolate incorporated with *A. muciniphila* demonstrated that more than 50% of the testers could not distinguish between a plain chocolate (control) and a probiotic-incorporated chocolate. Although the addition of probiotics leads to changes in the organoleptic properties of the chocolate matrix, the supplementary benefits of a probiotic chocolate overweigh these differences, being therefore more attractive to the consumer. In conclusion, a chocolate matrix with high cocoa content (dark chocolate) is a suitable carrier for *Bifidobacterium animalis* subsp. *lactis* BB-12® and *Akkermansia muciniphila*.

Keywords: Chocolate**;** *Bifidobacterium animalis* subsp. *lactis* BB-12®; *Akkermansia muciniphila*; Bioactivities; Viability

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List of Abbreviations

a_w – water Activity

CFU – Colony Forming Unit

EFSA – European Food Safety Authority

FAO/WHO – Food and Agriculture Organization/World Health Organization

FDA – Food and Drug Administration

GAE – Gallic Acid Equivalent

GIT – Gastrointestinal Tract

GRAS – Generally Regarded as Safe

IgA – Immunoglobulin A

IgE – Immunoglobulin E

NGP – Next Generation Probiotic

QPS – Qualified Presumption of Safety

SCFA – Short-chain Fatty Acids

TPC – Total Phenolic Compounds

VAP – Ventilator-Associated Pneumonia

WI – Whiteness Index

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Poster Presentation

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Vedor, R., Machado, D., Barbosa, J., Almeida, D., Andrade, J. C., & Gomes, A. M. (2022). Incorporation of the Next Generation Probiotic *Akkermansia muciniphila* in a chocolate matrix. Poster submitted to be presented in 5th Edition of ISEKI e-conference, 23rd - 25th November 2022.

Short Oral Communication

Vedor, R., Machado, D., Barbosa, J., & Gomes, A. M (2022). Chocolate funcional contendo o probiótico *Bifidobacterium animalis* subespécie *lactis* BB-12®. Short oral communication presented online in Microbiologia 2022, Lisboa, 17th - 19th October 2022.

1. Introduction

1.1. Probiotics

The history of probiotics is strongly linked to human history, as probiotics are highly associated with fermented foods, which were first mentioned around 10,000 years ago when humankind began practicing farming (Gasbarrini et al., 2016). However, modern probiotic history only dates to the early 20th century, with Ellie Mechnikov (Russian Scientist and Nobel Laureate). In his book "The Prolongation of life", he introduced the idea that bacteria present in the human gut played a role in adult human's life, more specifically in aging, hence his quote "death begins in the colon" (Metchnikoff, 1908.; Mizock, 2015). By 1965, scientists Lilly and Stilwell were using the term 'probiotic', a word of Greek origin translating to 'for life', to refer to secreted substances that stimulate the growth of others (Lilly & Stillwell, 1965; Mizock, 2015). The concept has been extensively explored and adjusted (Martín & Langella, 2019; Voss et al., 2022). Currently, probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014).

As previously mentioned, even though probiotics have been present for more than 10,000 years in the human diet, their popularity has only risen with the arrival of the 21st century (Sanders, Merenstein, et al., 2018). This was due to the more than 2,000 clinical trials which have proven probiotics' benefits to the host (Aponte et al., 2020). Therefore, understanding probiotics' mechanisms of action is required to understand probiotics' benefits (Figure 1.1). The mechanisms of action of probiotics are diverse, heterogeneous, and strain-specific, remaining not fully elucidated (Plaza-Diaz et al., 2019). Even so, four primary mechanisms of action have been explicitly proposed: i) interference with pathogens; ii) strengthening of the barrier function of the intestine epithelial layer; iii) immune system shaping; and iv) gut-brain axis interaction (see Figure 1.1; Sánchez et al., 2017). Additionally, the metabolic role of probiotics is of greater importance, as its responsible for several mechanisms such as synthesis of short chain fatty acids (SCFA) and vitamins (LeBlanc et al., 2017), biopeptides (Ahtesh et al., 2018), energy extraction (Álvarez-Arraño & Martín-Peláez, 2021) and bile acid conjugation with hypocholesterolemic effect (Pavlović et al., 2012).

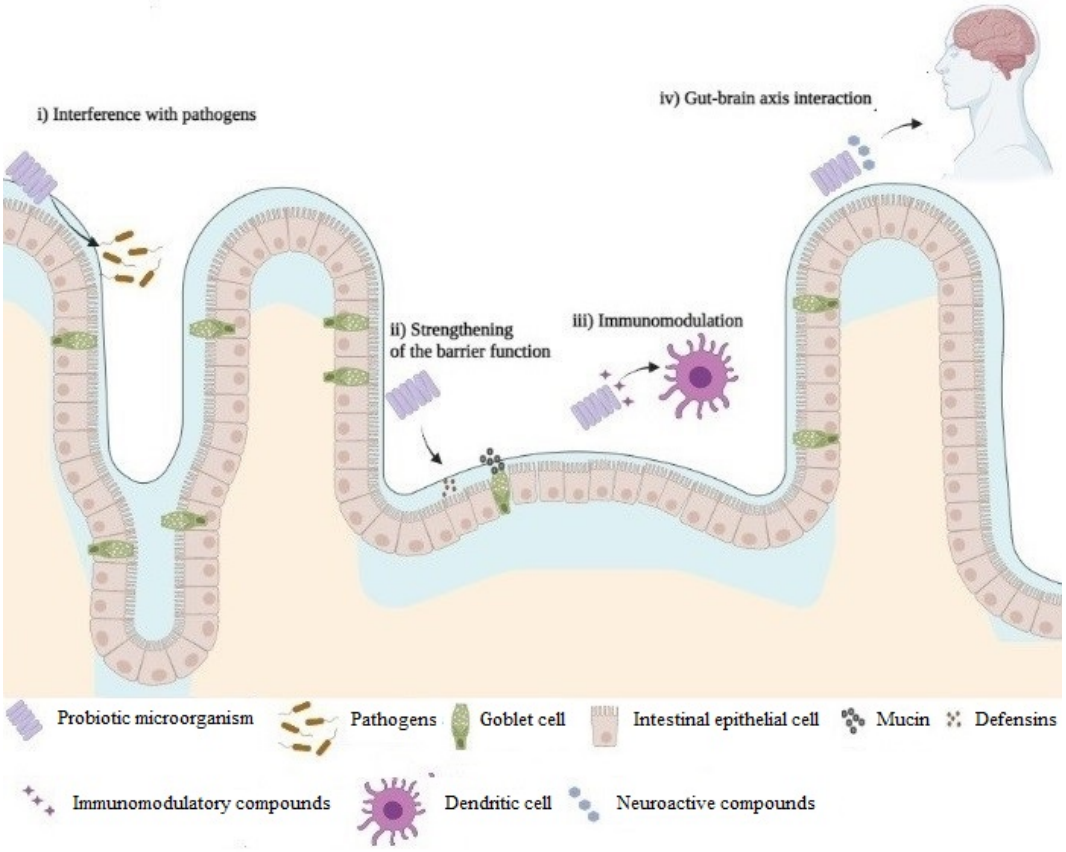


Figure 1. 1 Mechanisms of action of probiotics. This graphical representation shows the four main mechanisms by which probiotics exert beneficial health effects: i) interference with pathogens; ii) strengthening of the barrier function; iii) immunomodulation; and iv) gut-brain axis interaction. Representative scheme from Voss et al., 2022.

Probiotic microorganisms can be isolated from a variety of sources. Valuable sources for the isolation of potential probiotic strains include human gastrointestinal tract (Kim et al., 2018; Kirtzalidou et al., 2011; Ryan et al., 2008); maternal breast milk (Damaceno et al., 2021; Kirtzalidou et al., 2011); animal-origin foods, such as raw milk and fermented foods (Ragul et al., 2020; Reuben et al., 2020; Taye et al., 2021); and fermented plant-based foods (Lee et al., 2019; Oh et al., 2020). However, despite the great panoply of available probiotic sources, the isolated microbial strains must meet specific safety, functionality, and technological utility criteria to qualify as a probiotic (Binda et al., 2020; Zommiti et al., 2020). Firstly, and foremost, suitable identification and classification (genus, species, and, when applied, subspecies) are essential for the characterization of a probiotic strain. Nowadays, identification is mainly based on molecular techniques – particularly whole genome sequencing – as phenotypic methods alone are insufficient for correct identification (Seol et al., 2019). Secondly, an intensive characterization of the probiotic candidate, in terms of fitness and metabolic activity, should be performed to

understand its possible underlying mechanism(s) of action. Thus, the characterization of a microorganism should evaluate the following factors: survival under oro-gastrointestinal conditions, SCFA production (such as acetic or butyric acids), adhesion to mucus or intestinal epithelial cells, interaction with the immune system, resistance to digestive enzymes and bile, and antimicrobial activity (competitive exclusion and/or production of bacteriocins, organic acids, such as lactic acid, and hydrogen peroxide) (Galdeano & Perdigon, 2004; Kesen & Aiyegoro, 2018). A complete and correct identification and characterization of the probiotic allows the determination of whether the probiotic is safe for human consumption. In Europe, probiotic safety is determined by the European Food Safety Authority (EFSA), which has developed a list containing the species which are safe for human consumption – Qualified Presumption of Safety (QPS) (Binda et al., 2020).

Regarding the safety criteria for a probiotic, the strains should present the absence of acquired antimicrobial resistance genes, virulence factors such as toxins, invasion and/or adhesion factors, and the ability to form biogenic amines and D-lactate (Huys et al., 2013). Furthermore, according to the established definition of probiotic by Food and Agriculture Organization/ World Health Organization (FAO/ WHO), the probiotic should confer health benefits, and therefore at least one human trial must demonstrate that the probiotic confers said benefits (Sanders, Benson, et al., 2018). Lastly, it must be assured that the probiotic reaches the targeted areas, namely the human gut, in adequate amounts (10^6 to 10^{10} CFU/mL or CFU/g) (Binda et al., 2020; Kechagia et al., 2013).

At the commercialization level, the most applied probiotics in the food and pharmaceutical industries belong to the genus *Bifidobacterium* and the formerly known genus *Lactobacillus* (Zheng, Wittouck, Salvetti, Franz, Harris, Mattarelli, O'toole, et al., 2020), which are commonly termed classical probiotics (Almeida et al., 2020). Due to their safe application, the classical probiotics have been listed as Generally Regarded as Safe (GRAS) by the United States Food and Drug Administration (FDA) and QPS by the EFSA (Martín & Langella, 2019).

Despite the large array of commercially available probiotic products, the market's need for more diverse and target-specific products has risen in the last few years (Langella et al., 2019). Recent candidates derived from gut microbiota studies, such as *Akkermansia muciniphila* and the recently reclassified *Faecalibacterium* spp. (Sakamoto et al., 2022), have been pointed to as novel probiotic strains named Next Generation Probiotics (NGP) (Almeida et al., 2020). However, in this context, it is important to refer that several key

aspects, including effectiveness, safety, physiological, genomic, and metabolomic features of such microorganisms, remain not fully understood. Furthermore, before developing a practical application, other overlooked aspects such as strain propagation, storage stability, and delivery must also be the focus of research groups (Andrade et al., 2020).

The following sections, 1.1.1 and 1.1.2 focus on the probiotic strains used in the present thesis, specifically *Bifidobacterium animalis* subspecies *lactis* BB-12® and *Akkermansia muciniphila* DSM 22959. Thus, a descriptive analysis, in terms of physiological and metabolic features, health benefits, and applications, was performed for each bacterium.

1.1.1. *Bifidobacterium animalis* subspecies *lactis* (BB-12®)

Worldwide, *B. animalis* subsp. *lactis* BB-12® (from now on designated only by *B. animalis* BB-12®) is one of the most documented probiotics belonging to the *Bifidobacterium* genus (Jungersen et al., 2014). This bacterial strain, originated from Chr. Hansen's collection of dairy cultures, has been incorporated in infant formula (Holscher et al., 2012; Szajewska & Chmielewska, 2013), dietary supplements, and fermented milk products (including yogurt) (Chr Hanson, 2014). Microbiologically, *B. animalis* BB-12® is described as a lactic acid-producing, Gram-positive, rod shape, negative-catalase, anaerobic bacterium (Jungersen et al., 2014). Furthermore, this specific strain is highly attractive technologically due to its fermentation activity, high aerotolerance, suitable stability, and resistance to acid and bile. Beyond this, *B. animalis* BB-12® generally does not lead to dramatic changes in organoleptic properties (such as taste, appearance, and mouthfeel) when added to food (Cuffia et al., 2018; Mituniewicz–Małek et al., 2019). *Bifidobacterium animalis* BB-12® is also considered safe for human consumption since it has QPS status in Europe (EFSA, 2015) and was recognized as GRAS in the United States of America (FDA, 2022). In addition, several human clinical trials have proven the beneficial health effects of *B. animalis* BB-12® (see Table 1.1).

Table 1. 1 Clinical trial involving *B. animalis* subsp. *lactis* BB-12® and their human health benefits, published in 2021-2022

Target Condition	Key Finding	Reference
Tenseness and sleepiness	Military field training might lead to symptoms of sleep deprivation and psychological stress. The effect of supplementation on factors such as salivary immunoglobulin A (IgA), gastrointestinal symptoms, well-being indicators, and gut microbiota was evaluated. Participants were fed over 30 days a symbiotic ice cream containing *Lactobacillus acidophilus* LA-5 and *B. animalis* BB-12®. The intake of supplemented ice cream with *B. animalis* BB-12® improved the tenseness and sleepiness of healthy individuals undergoing intense physical training. The intake leads to decreased gastrointestinal symptoms due to a favorably modulated gut microbiota.	Valle et al., 2021
Alteration in the metabolism due to antibiotic intake	The administration of broad-spectrum antibiotics may lead to the dysregulation of the gastrointestinal tract, often resulting in antibiotic-associated diarrhea. The impact of *B. animalis* BB-12® supplementation throughout 14 days on the production of SCFA and microbiota composition was evaluated. The intake of *B. animalis* BB-12® leads to smaller decreases in the SCFA levels after antibiotic prescription. Probiotic intake lessens the changes in the microbiota composition. Another study evaluated the delivery efficacy of various matrices on the target condition, analyzing the effects on fecal microbiota, gut transit time, and SCFA. The results demonstrated no significant changes when consuming a *B. animalis* BB-12® supplemented as a smoothie compared with a capsule. Smoothie consumption leads to a higher relative abundance of *B. animalis* BB-12® in adults.	Ba et al., 2021; Merenstein et al., 2021

Respiratory Infections and Acute Gastroenteritis in children	Attending nurseries, kindergartens and schools may present a risk factor for developing diseases such as acute gastroenteritis and upper respiratory tract infections. The effect of the intake of *B. animalis* BB-12® in combination with *Enterococcus faecium* L3 on the development of the mentioned illnesses was evaluated in comparison with a control group. Probiotic intake significantly reduces the occurrence and duration of both syndromes while increasing salivary IgA levels.	di Pierro et al., 2021
Allergic Rhinitis	Probiotics have anti-inflammatory and immunomodulatory effects, leading to the possible prevention of an allergic response. The impact of the prophylactic intake of a probiotic mixture, which includes *B. animalis* BB-12® and *E. faecium* L3, on allergic symptoms was evaluated. The intake of *B. animalis* BB-12®, in combination with other probiotics, alleviates symptoms associated with allergic rhinitis in infants. This alleviation resulted from the modulation of the immune system through regulating IgE production. This improvement reduced the need for conventional therapy, including oral antihistamines and local corticosteroids.	Anania et al., 2021
Infant colic	The impact of *B. animalis* BB-12® intake on infantile colic over 6 weeks was evaluated. Oral supplementation of young infants with *B. animalis* BB-12® reduced crying time and ameliorated sleep during infant colic episodes. The observed effects were associated with *B. animalis* BB-12® capacity to modulate and regulate the microbiota.	Pourmirzaiee et al., 2021 Chen et al., 2021
Cholesterol	One of the suggested effects of probiotics is modulating the hosts' lipid metabolism by altering the intestinal microbiota. The effect of a probiotic-containing milk formula (*L. acidophilus* La5, *Lactobacillus casei* TMC, *B. animalis* BB-12®) on cholesterol levels was assessed. The intake of the probiotic-containing formula over 10 weeks improved gastrointestinal function through the	Chiu et al., 2021

	modulation of fecal movement and intestinal microbiota. The total cholesterol content decreased, allowing the control of hypercholesterolemia, and associated cardiovascular diseases.	
Immunity and Inflammation in elderly people	One of the consequences of aging is the deterioration of the immune system (immunosenescence). Probiotics have been suggested as a vehicle for the improvement of the immune system. The effect of intake over 12 months of two probiotics, *Lacticaseibacillus rhamnosus* GG (LGG) and *B. animalis* BB-12®, on immune biomarkers, such as plasma immune mediator concentrations and phagocytosis, was evaluated. Although no significant changes were observed in the analyzed components, the counts of both probiotics were higher in the testing group's feces than in the control.	Castro-Herrera et al., 2021
Thymus size and markers of infection in late infancy	The effect of probiotics on the immune system has been extensively explored; however, the impact on thymus size in late infancy is still poorly researched. The effect of probiotics on thymus size, C-reactive protein, and infections was assessed: children were fed a combination of LGG and *B. animalis* BB-12® or a placebo for 6 months. No significant differences were observed in thymus size between both groups; however, the probiotic-fed group demonstrated a higher thymus weight index. No significant changes were observed concerning the C-reactive proteins.	Larnkjær et al., 2021
Dental caries	Several factors may provoke dental caries; however, microorganisms constitute one of the main etiological factors. The effect of a probiotic curd (containing *L. acidophilus* LA5 and *B. animalis* BB-12®) on dental caries was evaluated. Salivary *Streptococcus mutans* counts showed no significant differences after a 7-day intervention for the study and control groups. After 24 days, significant differences were detected for *S. mutans* counts, suggesting that this probiotic curd could decrease salivary *S. mutans* levels.	Sakhare et al., 2021

Surgical Site Infections	The protective effect of probiotics in surgical site infections in multiple-trauma patients was assessed, focusing on the efficacy of a four-probiotic strategy (*L. acidophilus* LA5, *Lactiplantibacillus plantarum* UBLP-40, *B. animalis* BB-12®, and *Saccharomyces boulardii* Unique-28) on 100 trauma patients over 15 days. Only 36 incidents were recorded, with *Staphylococcus aureus* and *Acinetobacter baumanni* being the most common pathogens. A preventative strategy in multiple trauma patients is suggested to decrease the chances of post-operation infections.	Tsilika et al., 2022
Ventilator-Associated Pneumonia (VAP)	The role of a probiotic regimen as a preventative strategy for ventilator-associated pneumonia in ventilated multi-trauma patients was evaluated: a probiotic formula containing *L. acidophilus* LA5, *L. plantarum*, *B. animalis* BB-12®, and *Saccharomyces boulardii* was administrated twice for 15 days. Results show that the administration of probiotics leads to a reduced risk of VAP and sepsis. This preventative strategy reduced the stay in the intensive care unit and the hospital overall.	Tsilika et al., 2022

1.1.2. Akkermansia muciniphila

Akkermansia muciniphila belongs to the *Verrucomycrobia* phylum, representing around 0.5 to 5% of the bacteria in the human gastrointestinal tract (GIT) (de Vos, 2017; Png et al., 2010). This bacterial species is a crucial symbiont of intestinal microbiota since it can shape the host's immune responses and may participate in immune tolerance to commensal microorganisms (Derrien et al., 2011). Microbiologically, *A. muciniphila* is described as a Gram-negative, strictly anaerobic, oval-shaped mucin-degrading bacterium (Derrien et al., 2004). However, despite its reported strict anaerobic nature, recently, it was demonstrated that *A. muciniphila* exhibits a certain tolerance and resilience to aerobic environments, maintaining a high level of culturability (Machado et al., 2020). Furthermore, *A. muciniphila* presented a high survival rate when exposed to *in vitro* GIT conditions (Machado et al., 2020). Thus, the aerotolerance, conjugated with resistance to GIT conditions, makes *A. muciniphila* very attractive from a technological point of view. Concerning its safety profile, *A. muciniphila* is commercialized as a dietary supplement called "Pendulum Akkermansia" in the USA (*Pendulum*, 2022). In Europe, the EFSA's Scientific panel published in 2020 an updated QPS list, in which *A. muciniphila* was evaluated for the first time. Although profiles are promising several correlational studies that require further understanding keep *A. muciniphila* from being included in this QPS list (Koutsoumanis et al., 2020). Nevertheless, this assessment did not take into consideration recent findings showing that the antimicrobial susceptibility profile of *A. muciniphila* DSM 22959 meets the safety criteria required to be considered safe for human consumption (Machado et al., 2022). In addition to its technological advantages and safety profile, several *in vivo* studies have demonstrated that *A. muciniphila* triggers a remarkable panoply of positive health effects, supporting their application in the treatment and prevention of various disorders (see Table 1.2).

Table 1. 2 *In vivo* studies involving *A. muciniphila* and their health benefits, published in 2021-2022

Target Conditions	Key Findings	Reference
Acute Colitis	*A. muciniphila* has been associated with a positive effect on diseases such as obesity and diabetes. However, its effect on inflammatory bowel diseases (chronic intestinal dysbiosis) has been poorly explored. The impact of *A. muciniphila* administration on the development of acute colitis was assessed. Probiotic intake ameliorated symptoms by contributing to body weight loss, colon length shortening, and colon histological inflammatory score. In the mice models, gut dysbiosis was observed due to the dextran sulfate sodium-induced acute colitis. *A. muciniphila* led to normalizing the gut microbiota, differentiation of Tregs, and increased production of SCFA. The underlying action mechanism lies in the activation of NLRP3.	Qu et al., 2021
Boost of antiaging and anticancer metabolites	*A. muciniphila* is linked with signs of health, leanness, and fitness, as several experiments have already demonstrated the positive effects of *A. muciniphila* on obesity and diabetic mediation, aging, inflammation, and immunosurveillance of cancer. The time-dependent effects of the administration of live and pasteurized *A. muciniphila* on mice were evaluated, and it was shown that *A. muciniphila* affected the metabolism by increasing polyamines such as spermidine, SCFA, 2-hydroxybutyrate, and bile acids in the gut and liver.	Grajeda-Iglesias et al., 2021
Atopic dermatitis	Atopic dermatitis is a known inflammatory skin disease often linked with gut health due to the gut-skin axis. Two NGPs, namely *F. prausnitzii* and *A. muciniphila*, play a crucial role in the pathogenesis of this disease. The potential beneficial effect of these probiotics on atopic dermatitis was assessed using	Lee et al., 2022

	a mice model. The intake of *F. prausnitzii* and *A. muciniphila* improved the markers related to atopic dermatitis, namely dermatitis score, scratching behavior, and serum Ig E levels. This intake also balances the Th1 and Th2 immune responses.	
Liver Injury	Liver fibrosis is the number one cause of death among patients with chronic liver disease. The benefits of consuming live and pasteurized *A. muciniphila* in preventing liver fibrosis were evaluated in a mice model. Both preparations led to a higher protective effect against hepatic stellate cell activation, improved serum biochemical and inflammatory cytokines levels, and improved liver and colon histopathological damage. Oral administration of *A. muciniphila* contributed to restoring the gut microbiota. Another study assessed a mouse model's exact impact of high-fat diet-induced liver fibrosis. The same results were obtained, proving that *A. muciniphila* leads to improved gut health due to strengthening the epithelium and intestinal integrity and inhibiting liver inflammation.	Keshavarz Azizi Raftar et al., 2021; Raftar et al., 2022
Influenza Virus Infection	Infection by the influenza virus may alter the gut microbiota composition. Additionally, the severity of the disease is highly linked to the gut microbiota. The consequences of the pathogenesis on the gut microbiota were evaluated, along with the determination of potential anti-influenza bacteria. Results indicated that the growth of *A. muciniphila* was promoted by influenza infection. Additionally, the supplementation of pasteurized *A. muciniphila* reduces weight loss and mortality and promotes anti-inflammatory and immunoregulatory properties.	Hu et al., 2021

***Clostridioides difficile* Infection**	*Clostridioides difficile* is a pathogen known for causing nosocomial infections. One of the leading causes of this pathogenesis is the antibiotic-induced dysbiosis of the intestinal microbiota. The effects of *A. muciniphila* in a *C. difficile* infection were evaluated in a mice model. Supplementation with *A. muciniphila* prevented weight loss and histological injury in the colon and alleviated symptoms of inflammation. The underlying mechanism is based on the increased production of SCFA, the maintenance of bile acids, and the modulation of the gut microbiome.	Wu et al., 2022
Muscular Atrophy	Muscular atrophy is characterized by the loss of muscle mass and strength caused by numerous factors such as injury, aging, metabolic disorder, or chronic conditions. The effect of *A. muciniphila* and *F. prausnitzii* intake was assessed in a mouse model with muscular atrophy. Probiotic supplementation improved grip strength but did not result in muscle mass gain.	Byeon et al., 2022
Diabetes mellitus	The protective effect of live and pasteurized *A. muciniphila* and the correspondent protein Amuc_1100 against diabetes mellitus was analyzed in a mice model. The group supplemented with *A. muciniphila* reduced its body mass gain and plasma TNF-α levels. The administration increased the number of goblet cells and mucin secretion. To a certain extent, *A. muciniphila* was also influential in restoring gut barrier function. No significant effects were observed on the gut microbiota structure.	Deng et al., 2022
Periodontal and systemic inflammation	The oral administration of live, pasteurized *A. muciniphila* or Amuc_1100 led to decreased *Porphyromonas gingivalis*-induced periodontal destruction and inflammatory infiltrate in lean and obese mice.	Mulhall et al., 2022

1.2. Chocolate as an opportunity for a merger between science and the food industry

Chocolate is constituted by a fatty matrix, dubbed cocoa butter, in which the cocoa and sugar particles are incorporated. This constitution leads to its unique characteristic of being solid at room temperature yet being able to quickly melt in the mouth (Montagna et al., 2019). Its variable composition segregates and classifies this food product into defined categories with specific properties (Montagna et al., 2019). The primary categories are dark, milk, and white chocolate, which differ in the formulation's cocoa solids, milk fat, and cocoa butter contents (Petyaev & Bashmakov, 2017). According to the Directive 2000/36/EC of the European Parliament and of the Council of 23 June 2000 related to cocoa and chocolate products intended for human consumption, to characterize a product as chocolate, it must "be obtained from cocoa products and sugars which contains not less than 35% total dry cocoa solids, including not less than 18% cocoa butter and not less than 14% of dry non-fat cocoa solids". In turn, milk chocolate is designated as “the product obtained from cocoa products, sugars and milk or milk products, which, subject to contains not less than 25% total dry cocoa solids" (DIRECTIVE 2000/36/EC, 2000).

Worldwide, the average chocolate consumption rate is estimated at 0.9 kg per capita per year (Tan et al., 2021). Specifically, this consumption rate in Portugal is valued at 2.0 kg per capita per year (Matos et al., 2018). The popularity of chocolate is mainly due to its ability and potential to sensorially arouse pleasure and trigger positive emotions on a psychological level (El-kalyoubi et al., 2011; Konar et al., 2016). However, chocolate is sometimes seen as a harmful food for human health, and nowadays, there is a change in consumers' consumption patterns, with health at the center of the concern.

The increase in cardiovascular, gastrointestinal, and diet-related diseases has led to an increased consumer interest in the ingredients present in the food products they eat (Cencic & Chingwaru, 2010). This concern is characterized by an appreciation of products connoted as functional foods. Functional food is “a conventional food or food similar in appearance to traditional food, that is part of a regular diet and has proven health-related benefits and/or reduces the risk of specific chronic diseases above its essential nutritional functions” (Hasler, 2000). As a result, four core aspects are highlighted in the concept of functional food: health benefits, nature of the food, level of function, and consumption pattern (Hasler, 2000).

In the last decades, functional foods have been gaining prominence in the food market, mainly due to the extensive number of products that have been emerging in this field (Belščak-Cvitanović et al., 2015). Within this context, the confectionery industry, with particular emphasis on the cocoa and chocolate industries, has been undergoing dynamic changes as there is an increase in demand and requests for healthier or functional chocolates (Rezende et al., 2015).

With this, an opportunity arises to improve the nutritional value of highly consumed and appreciated foods, such as chocolate (Rezende et al., 2015). In this context, functional confectionery is introduced and defined as "a confectionary item that has undergone the addition, removal or replacement of common confectionery ingredients with an ingredient that fulfills a specific physiological function or offers a potential health benefit" (Pickford & Jardine, 2000).

Several studies have suggested that chocolate, in particular dark chocolate, has the potential to confer benefits to human health due to the presence of a grand panoply of bioactive compounds (Araujo et al., 2016). The cocoa bean itself is responsible for more than 300 of the chemical compounds present in chocolate. The bioactive compounds can be classified as the following: polyphenols, in which flavonoids and non-flavonoids are included; methylxanthines, which comprise theobromine and caffeine; and minerals, such as magnesium, iron, and zinc, among others (Petyaev & Bashmakov, 2017). All the above-mentioned bioactive molecules benefit the human body; however, studies suggest flavonoids are essential in the prevention of cardiovascular diseases (Mangels & Mohler, 2017; Petyaev & Bashmakov, 2017). Furthermore, these particles are in higher quantities in dark chocolate, as this type of chocolate is richer in cocoa solids (flavonoids account for 12-18% of dry weight cocoa beans (Lik Hii et al., 2009) compared to white and milk chocolate). As a result, dark chocolate has a higher flavonoid content and, therefore, higher antioxidant activity (Petyaev & Bashmakov, 2017).

Various favorable bioactive compounds in chocolate present an opportunity to develop chocolate-based products, which act as nutraceutical products (Petyaev & Bashmakov, 2017). Table 1.3 shows some health benefits related to chocolate consumption.

Table 1. 3 Beneficial effects of chocolate consumption

Target Condition/ Body Part	Key Findings	Reference
Skin	Some studies suggest that cocoa has photoprotective effects. Daily intake of chocolate may lead to the improvement of blood circulation, oxygen saturation, skin density, and hydration. Cocoa supplementation may result in the reduction of wrinkles and skin roughness.	Calzavara-Pinton et al., 2019; Yoon et al., 2016
Depression and Anxiety	Consumption of chocolate reduces the probability of developing anxiety- and depression-like symptoms. The intake of chocolate is highly associated with improving one's mood, at least short term. This effect is believed to be caused by psychoactive components, such as anandamide analogs, and biogenic amines, such as phenylethylamine – a neuromodulator involved in mood regulation. Dark chocolate is more effective than milk chocolate due to its high flavonoid concentration, which improves inflammation (inflammation is highly associated with poor mood).	Jackson et al., 2019; F. P. J. Martin et al., 2012; Pase et al., 2013
Bone health	The consumption of chocolate during pre-teen and teenage years leads to higher bone density and, consequently, higher longitudinal bone growth. Unsweetened and dark chocolate has been proven to have greater efficacy when it comes to the preservation of bone health.	Seem et al., 2019
Chronic Fatigue Syndrome	Several studies suggested that chocolate consumption alleviated fatigue symptoms in patients suffering from other chronic diseases such as multiple sclerosis. This capacity is highly linked with high flavonoid content, which results in antioxidant and anti-inflammatory activities.	Coe et al., 2017, 2019; McDermott et al., 2020

		Sathyapalan et al., 2010
Allergies	A study conducted with university students showed that moderate cocoa intake leads to a diminishment in allergy-like symptoms. Pre-clinical trials have supported this theory, as cocoa intake is associated with regulation and modulation of T cell functions, which is linked to systemic and gut antibody synthesis. This modulation leads to a reduction in IgE synthesis, which is responsible for allergic reactions.	Pérez-Cano et al., 2013; Rodríguez-Lagunas et al., 2019
Aging	Studies suggest that the daily intake of cocoa-rich supplements leads to fewer hospitalization and deaths among the elderly population. Regular consumption of chocolate results in enhanced cognitive performance and verbal fluency, decreased insulin resistance and lipid peroxidation, increased neurovascular spacing, and improved visual-spatial attention. Daily chocolate intake leads to enhanced cognitive and memory function, improving life quality among the elderly.	Mastroiacovo et al., 2015; Mostofsky et al., 2010; Munguia et al., 2019
Cognitive Functions	Several studies have proven that the consumption of cocoa/ chocolate improves cognitive function, in particular functions such as attention, working memory, processing speed, and visual search efficiency. Additionally, its intake positively affects attenuated mental fatigue. These studies have both been applied to young and elder generations.	Sumiyoshi et al., 2019 Field et al., 2011
Diabetes	Recent findings have proven that regular intake of chocolate leads to a reduced risk of developing type 2 diabetes. In diabetes patients, cocoa consumption affects factors such as blood pressure, lipoprotein status, arterial stiffness, insulin levels, and resistance. As a result, cocoa intake reduces the risk of developing other cardiovascular diseases in diabetes patients. The critical mechanisms involved in this process include the	Álvarez-Cilleros et al., 2020; Crichton et al., 2016; Curtis et al., 2013;

	modulation of proteins, which interfere in insulin signaling pathways, inflammation, oxidative stress, and microbiota.	
Obesity	Contrary to popular belief, chocolate consumption is not directly correlated with extreme gain in weight. Some studies suggest that regular cocoa intake is associated with lower body mass index and body weight, as well as lower levels of triacylglycerols and oxidative stress-related factors. More importantly, the intake of high cocoa chocolates leads to ameliorating obesity-related symptoms such as improvement in blood pressure, glucose and insulin levels, dyspepsia, and gastrointestinal manifestations. Obesity is associated with dysbiosis, and the high cocoa content helps to regulate the gut microbiome. Dark chocolate consumption has been proven to help with satiety, decreasing the need for something sweet.	Frisse et al., 2012; Massolt et al., 2010; Nickols-Richardson et al., 2014; Ribeiro Vieira et al., 2017
Cardiovascular Disease	Numerous studies have proven a positive correlation between regular chocolate consumption and decreased risk of cardiovascular diseases. Chocolate intake reduces the risk of developing cardiovascular diseases such as stroke, coronary artery disease, myocardial infarction, and ischemic heart disease. Also, chocolate interferes with cardiovascular risk factors: endothelial function, blood pressure, lipid metabolism, and vascular stiffness.	Buitrago-Lopez et al., 2011; Kwok et al., 2015; M. Á. Martin & Ramos, 2021; Ren et al., 2019

1.3. Chocolate: a valuable food matrix for probiotic delivery

Chocolate incorporating probiotics may be a valuable candidate for novel functional foods due to the combined health benefits of the probiotics and chocolate bioactive compounds (Faccinetto-Beltrán, Gómez-Fernández, Santacruz, et al., 2021; Silva et al., 2017). Indeed, chocolate has been proposed as a suitable carrier to deliver probiotics, either in their free or encapsulated forms (see Table 1.4), as an essential alternative to fermented dairy products (Hossain et al., 2021). However, the incorporation of certain probiotic bacteria, such as *B. animalis* subsp. *lactis* BB-12® and *A. muciniphila* into chocolate matrices remains limited (Hossain et al., 2022; Marcial-Coba et al., 2019). Given their fastidious and anaerobic nature, these two probiotics have yet been incorporated as free cells in chocolate. Together with the increasing demands for functional chocolates, this creates an opportunity to innovate the probiotic market as well as the confectionery and food industries.

Table 1. 4 Types of chocolate incorporated with probiotics

Strains	Probiotic Form	Chocolate Type	Key Findings	Reference
L. acidophilus; L. casei; L. rhamnosus* LGG; *Bifidobacterium animalis* ssp. *lactis	Free cells	Milk chocolate	The effect of different ratios of sucrose/D-tagatose and different probiotics on milk chocolate properties, such as biochemical and microbiological characteristics, residual sugar, color, and sensory attributes, was assessed. Probiotic viability highly depends on the used strain and sweetener (sucrose vs. D-tagatose). The highest viability was observed in the chocolate containing *L. rhamnosus*, followed by *L. casei* in D-tagatose. Sensory analysis revealed that the probiotic choice induced statistically significant differences in texture. *Lactobacillus* strains mostly fermented D-tagatose while *B. lactis* did not consume the added sugars. D-tagatose could be a great candidate for substituting sugar in milk chocolate, enhancing its functional properties.	Rouhi et al., 2015
***Lactobacillus helveticus* CNCM I-1722 and *Bifidobacterium longum* CNCM I-3470**	Freeze-dried microencapsulated powder	Milk and dark chocolate	The protective power of chocolate as a possible carrier of two probiotic strains for oral delivery was assessed. Probiotic viability was superior in the chocolate matrix compared to the milk one (90% and 30% viability, respectively). Under gastrointestinal conditions, the chocolate matrices conferred superior protection, and when comparing both chocolate matrices, milk chocolate has higher protection power. This higher protection power in milk chocolate compared to dark chocolate was proposed to be mainly due to the polyphenol	Possemiers et al., 2010

			content's antimicrobial effects, which were likely higher in dark chocolate. Hereby, it was shown that chocolate is a novel candidate for the coating of probiotics as it protects the bacteria from environmental stress conditions in the gastrointestinal tract.	
***L. acidophilus* La-14 ATCC SD5212 and *Lactobacillus paracasei* Lpc-37 ATCC SD5275**	Free cells	White chocolate	The effect of inulin on the viability of two different probiotics was evaluated, and chocolate properties in sugared and sugar-free chocolates were assessed. After 90-day storage, *L. acidophilus* presented higher viability in all tested chocolates. The choice of probiotic strain impacts microbial viability in white chocolate. The addition of inulin probiotics impacted white chocolate's rheological properties, color, and texture.	Konar et al., 2018
***Lactobacillus brevis* subsp. *coagulans* (Labre) FERM BP-4693** ***Lactobacillus brevis* NTM003 (NTM003) NITE BP-1634**	Freeze-dried cells	Milk Chocolate	A method to improve acid tolerance of probiotic bacteria was developed, aiming to improve probiotic effects in the gastrointestinal tract. Probiotic delivery in chocolate was proven more effective than traditional delivery systems such as powder and yogurts. It was hypothesized that the different tolerance levels are due to the water content. This delivery system effectively protected cell viability from the stress of the gastrointestinal tract. Chocolate protection also positively impacted the enzymatic activities of probiotics.	Yonejima et al., 2015

***L. rhamnosus* GG*; L. paracasei* F19*; L. casei* DG*; Lactobacillus reuteri* DSM17938**	Dissolved freeze-dried cells in UHT milk	Dark Chocolate (50% - 85%)	One of the still unanswered questions concerning the incorporation of probiotics in chocolate is the effect of the polyphenol content on bacterial growth. This study aimed to assess the viability of different probiotic strains in dark chocolate. Dark chocolate is a viable choice as a carrier of probiotic bacteria, although viability was highly dependent on the bacteria and the inoculation technique (the pre-inoculation in a milk matrix led to significant viability loss). The best viability and sensory quality results were obtained for the chocolates inoculated with LGG and F19. Lyophilization of the probiotic cells has a protective effect against the polyphenol content of dark chocolate.	Succi et al., 2017
***L. acidophilus* LA3 *and B. animalis subsp. lactis* BLC1**	Free cells	Semisweet chocolate	The potential of semisweet chocolate as a vehicle for two probiotic strains was evaluated. Incorporating the probiotic cells in semisweet chocolate is a viable option for probiotic delivery, as cell viability was maintained for 125 days. Chocolate conferred protection to the cells throughout the exposure to the GIT. This protection might be due to the slightly acidic pH, low water activity, and high fat and phenolic content in semisweet chocolate. Concerning sensorial analysis, the incorporated chocolate was well received by the panelists.	Silva et al., 2017
A. muciniphila* and *L. casei	Microencapsulated cells in xanthan/gellan gum gel matrix	Dark chocolate	Various factors, such as storage and GIT passage, may affect probiotic viability. *A. muciniphila* is highly sensitive to oxygen, needing a protective matrix for its delivery. This study assessed the viability of *A. muciniphila* and *L. casei* cells when embedded in dark chocolate after microencapsulation in a	Marcial-Coba et al., 2019

			xanthan/gellan gum matrix. For both probiotics *A. muciniphila* and *L. casei*, cell viability was maintained over a 60-day storage period in an anaerobic atmosphere at 4ºC, with a loss of less than 1log CFU/g. This protection was also evident in gastric transit. The protective power was higher for *A. muciniphila* cells than for *L. casei* cells compared to control assays: *A. muciniphila* embedded in chocolate increased its viability by 1.8 log CFU/mL, while *L. casei* viability only improved by 0.8 log CFU/mL.	
Lactobacillus plantarum 299v* and *L. rhamnosus GG	Free Cells	Milk chocolate	This study aimed to develop a chocolate matrix that acts simultaneously as a vehicle for Omega-3 (ω3) polyunsaturated fatty acids and probiotics. Fish oil was incorporated as a source of polyunsaturated fatty acids. The incorporation of fish oil and probiotics led to a decrease in the whiteness index and water activity. The combination of both did not change chocolate's acceptability by the public, although using a higher concentration of fish oil did not maintain adequate physiochemical and sensory properties.	Faccinetto-Beltrán, Gómez-Fernández, Orozco-Sánchez, et al., 2021
L. plantarum 299v* and *L. acidophilus La3	Free cells	Milk chocolate	This work acts as a continuation of the previous one. The effects of substituting sugar in milk chocolate for alternative sugar sweeteners were assessed, in this case, isomalt in combination with stevia. The chocolate containing isomalt + stevia, fish oil, and probiotics showed high consumer acceptability compared to those containing probiotics and fish oil. The values	Gómez-Fernández et al., 2021

			of chocolate properties, such as water activity and hardness, were maintained similarly throughout all tested conditions.	
L. casei 431	Free cells	Milk chocolate	The impact of various sweeteners on the viability of the probiotic *Lactobacillus casei* 431 was assessed. The highest viability results were obtained for the chocolate produced with sucrose and stored at 4ºC. Sucrose-free chocolate had better sensory quality and viscosity scores. Sugared and sugar-free chocolates are suitable probiotic vehicles and can be stored at room temperature as bacterial viability is maintained.	Rad et al., 2020
L. plantarum	Encapsulated cells	Dark Chocolate	Encapsulated *L. plantarum* 564 and commercial *L. plantarum* were inoculated in dark chocolate to assess their viability. Viability is maintained to the required levels (10^6 CFU/g) for up to 180 days. The chemical composition of the chocolates was evaluated, and no statistical differences were observed compared to control chocolate. The addition of probiotics did not affect the organoleptic properties of chocolate.	Mirković et al., 2018
Bacillus coagulans	Lyophilized cells	Dark Chocolate	The viability of *B. coagulans* in a dark chocolate matrix was assessed and its biochemical properties, such as polyphenols content, were determined. The viability of the probiotic was maintained during storage, and no significant viability losses were observed after submitting the sample to the *in vitro* GIT model. Adding *B. coagulans* did not affect water activity, pH, polyphenol content, and sensory quality.	Kobus-Cisowska et al., 2019

L. plantarum LRCC5193	Lyophilized cells	Milk chocolate	*L. plantarum* LRCC5193 isolated from Kimchi was incorporated in a chocolate matrix to study its viability during storage and gastrointestinal tract passage. Higher viability was obtained during the GIT when the chocolate matrix protects the probiotic cells.	Lim et al., 2018
L. acidophilus NCFM ® and Bifidobacterium lactis HN019	Encapsulated cells	Milk and dark chocolate	The study evaluated the viability of two different probiotic bacteria incorporated in milk and dark chocolate (57% and 72% cocoa content, respectively) during storage and GIT passage. One log cycle reduction was observed for both probiotics after one month of storage, and after 14 months of storage, viability was kept stable at a temperature of 15ºC. Compared with other matrices such as dairy and juice, chocolate showed higher protective power during GIT passage.	Klindt-Toldam et al., 2016

Note[1]: Numerous *Lactobacillus* species mentioned above have been recently classified, according to Zheng, Wittouck, Salvetti, Franz, Harris, Mattarelli, O'Toole, et al. (2020). Hereby, the species nomenclature was referred to as in the original papers to simplify the reading. The corresponding nomenclature is as follows (the former nomenclature appears first, followed by the new name):

Lactobacillus casei = Lacticaseibacillus casei

Lactobacillus helvictus = Lactobacillus helvictus

Lactobacillus paracasei = Lacticaseibacillus paracasei

Lactobacillus rhamnosus = Lacticaseibacillus rhamnosus

Lactobacillus plantarum = Lactiplantibacillus plantarum

Lactobacillus brevis = Levilactobacillus brevis

Lactobacillus reuteri = Limosilactobacillus reuteri

Lactobacillus salivarius = Ligilactobacillus salivarius

Lactobacillus acidophilus = Lactobacillus acidophilus

Note[2]: Recently, the *Faecalibacterium* strains were reclassified based on genome, phenotypic and chemotaxonomic traits. Hereby, three novel species have been proposed *Faecalibacterium duncanie* (= *Faecalibacterium prausnitzii*), *Faecalibacterium hatorri* and *Faecalibacterium gallinarum* (Sakamoto et al., 2022).

The introduction of a new chocolate in the industry requires a thorough characterization of said matrix, particularly, when proposing the incorporation of probiotic bacteria in the chocolate matrix. First and foremost, when producing a probiotic product, one of the most important traits to be evaluated, is the survivability and stability of the chosen probiotic in the proposed matrix during a storage period (Silva et al., 2017; Succi et al., 2017). In other words, cell viability during a defined storage period should be assessed, to ensure that the delivery matrix guarantees the minimum required levels of viable cell numbers (10^6-10^7 CFU/g or CFU/mL) for a probiotic product to exert its health benefits (Imlay, 2013; Kechagia et al., 2013). Additionally, the matrix should provide sufficient protection against the harsh conditions of the human gastrointestinal tract (GIT) to guarantee that the minimum required viable cell numbers reaches the intestine for beneficial action to occur (Naissinger da Silva et al., 2021; C. S. Ranadheera et al., 2019; D. M. D. Rasika et al., 2020). *In vitro* digestion models are the most common practice in the industry to simulate the GIT conditions and to evaluate structural changes, digestibility, viability, and compound release (Hur et al., 2011; Jacobsen et al., 2020; Naissinger da Silva et al., 2021).

As mentioned earlier, chocolate, particularly dark chocolate, confers benefits to human health as it contains more than 300 chemical bioactive compounds (Araujo et al., 2016). Hereby, emphasis goes to the high flavonoid content of dark chocolate, which results in a high antioxidant activity (Petyaev & Bashmakov, 2017). Additionally, dark chocolate presents antidiabetic properties, as it improves insulin sensitivity, which consequently reduces insulin resistance (Samanta et al., 2022). Moreover, dark chocolate grants antihypertensive activity, as it lowers the occurrence of high blood pressure by enhancing the circulation of nitric oxide (Samanta et al., 2022). Considering these properties, it's extremely important to analyze whether the addition of probiotics influences these bioactivities positively or negatively.

The development of a protective food requires the assessment of factors, which influence food quality and food safety, such as pH and water activity (a_w). For instance, chocolate's natural pH ranges from 5.6 to 6.8. Hereby, it is important to evaluate whether the addition of probiotic strains significantly alters the pH values. Ideally, this addition would not alter the pH values range, indicating that the added probiotic strains are not metabolically active, which benefits the preservation process of food products throughout a storage period (Silva et al., 2017; Succi et al., 2017). Additionally, water activity is an important factor concerning food microbial contamination. It is important to guarantee that the

addition of a probiotic strain does not alter the a_w values and therefore does not trigger the growth of pathogenic/contaminant microorganisms (Beuchat et al., 2013).

Lastly, factors related to sensory analysis should be evaluated when producing a food product. Hereby, it is important to assure that the addition of a probiotic strain to the chocolate matrix does not alter properties such as the overall quality aspect and the texture significantly. In other words, the addition of probiotic bacteria should not worsen the pleasureful experience a consumer has, when consuming a food product such as chocolate.

1.4. Aim of the work

Bifidobacterium animalis subspecies *lactis* BB-12® and *A. muciniphila* DSM 22959 are promising probiotic candidates for the incorporation in a chocolate matrix, given their potential in the treatment and prevention of metabolic and inflammatory diseases.

Founded on the above groundwork, the main goal of the present thesis work is to develop a chocolate matrix that acts as a probiotic delivery system for *Bifidobacterium animalis* subsp. *lactis* BB-12® and *Akkermansia muciniphila*. Therefore, it is important to identify the most adequate chocolate matrix for the incorporation of each probiotic strains, either *B. animalis* BB-12® or *A. muciniphila*, based on cell viability during aerobic storage and bioactivities, and evaluate their stability when exposed to GIT conditions, physico-chemical properties, and sensory attributes. Bearing in mind the main goal of the thesis work, the study was divided into two parts with different specific objectives:

Part I – Identification of the most appropriate chocolate matrix based on cocoa content

i) Assess *B. animalis* subspecies *lactis* BB-12® and *A. muciniphila* DSM 22959 cell viability in three different cocoa concentrations chocolates over 28 days of aerobic storage;

ii) Evaluate the evolution of pH, total phenolics content and overall aspect of the different probiotic chocolates over 28 days of aerobic storage;

iii) Analyse the relationships between cocoa content, probiotic addition and antioxidant, antidiabetic, and antihypertensive activities of chocolates stored for 28 days under aerobic storage

Part II – Detailed characterization of the best performing chocolate matrices for incorporation of each of the probiotic strains

i) Characterize the sensory attributes of a 70.5% *A. muciniphila* incorporated chocolate matrix

ii) Assess *B. animalis* subspecies *lactis* BB-12® and *A. muciniphila* DSM 22959 cell viability in each of the most adequate chocolate matrix throughout manufacture;

iii) Measure pH, water activity, surface color and texture chocolates upon manufacture;

iv) Determine the impact of the chocolate matrix on survival of *B. animalis* BB-12® and *A. muciniphila* when exposed to *in vitro* simulated GIT conditions;

Figure 1.2 offers a summary of the thesis framework, which is divided into four main sections, including the above detailed experimental work distributed over parts I and II, supported by an exploratory section that covers the state-of-art on *B. animalis* subspecies *lactis* BB-12® and *A. muciniphila*, detailing their health beneficial effects, and a detailed description on chocolate composition, including its health benefits and examples of already explored topics on probiotic chocolates. Finally, a critical discussion of the achieved results was performed. The thesis is completed by a fifth section providing concluding remarks and proposals for future work.

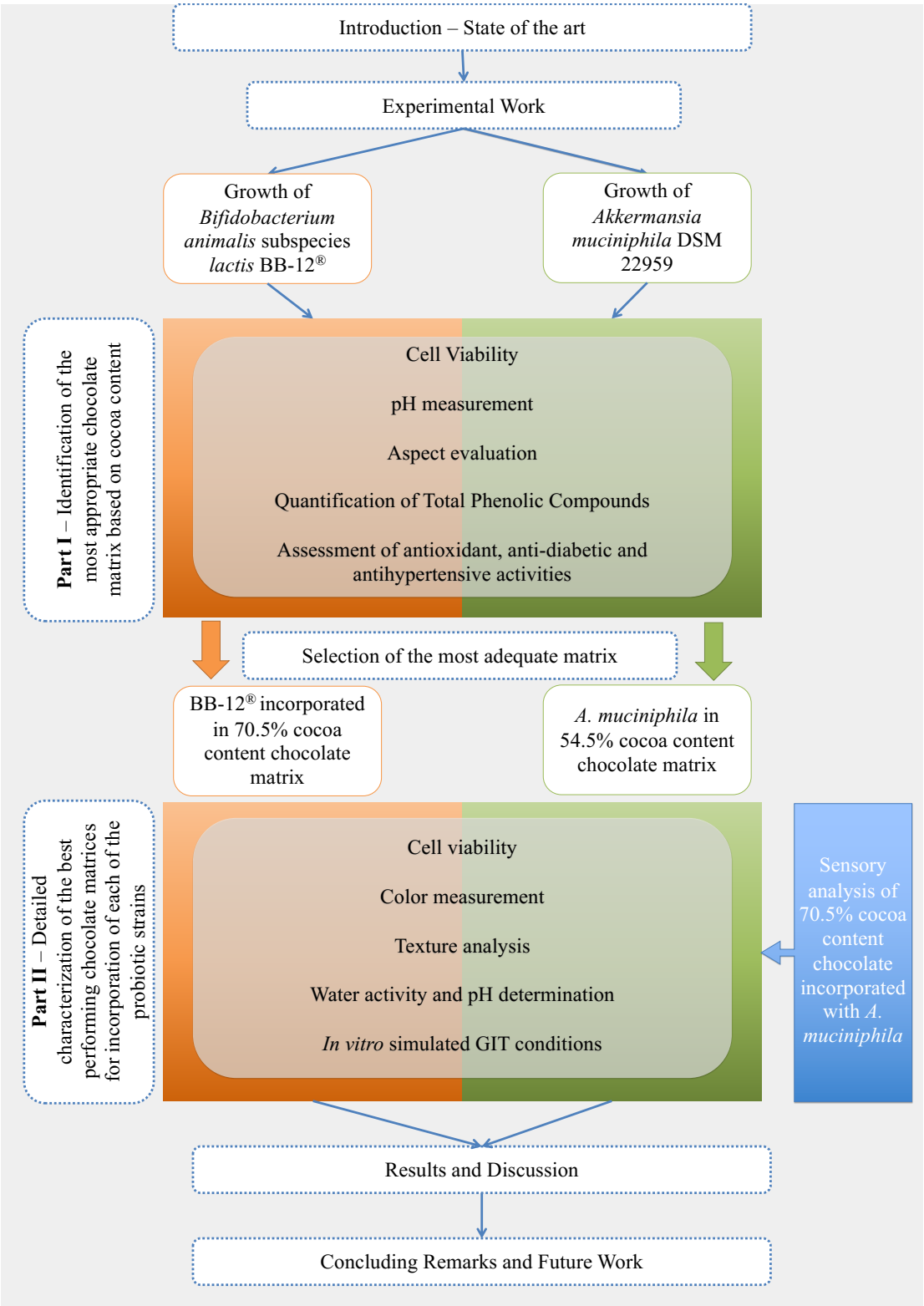


Figure 1. 2 Schematic flow chart of thesis outline

2. Material and Methods

2.1. Bacterial strains and growth conditions

Akkermansia muciniphila DSM 22959 obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and *Bifidobacterium animalis* subspecies *lactis* BB-12® from Chr. Hansen collection (Hoersholm, Denmark) were used in the present study. For long-term storage, the bacterial strains were kept frozen at -80ºC in appropriate media with 20% (v/v) glycerol (Fisher Chemical, Loughborough, United Kingdom), namely: PYG broth supplemented with 0.05% (w/v) mucin (Sigma-Aldrich, St. Louis, Missouri, USA) for *A. muciniphila* DSM 22959 [PYGM media composition following DSMZ recommendations (DSMZ, 2020) except that no resazurin was added] and de Man Rogosa and Sharpe (MRS) supplemented with 0.05% (w/v) of L-cysteine·HCl (Alfa Aesar, Kandel, Germany) for *B. animalis* subsp. *lactis* BB-12®. For each experiment, a glycerol stock of each bacterial strain was thawed and grown in appropriate broth for 24h at 37°C under anaerobic conditions (85% N_2, 5% H_2, and 10% CO_2) achieved using an anaerobic incubator (Whitley A35 HEPA anaerobic workstation, Bingley, United Kingdom). Next, in the case of *A. muciniphila* DSM 22959, at least two subsequent culturing steps using the same growth conditions were performed, with a final incubation volume of 1 L of PYGM broth with 10% (v/v) of cell inoculation, to obtain a higher biomass yield. Then, *A. muciniphila* cells were harvested by centrifugation (Sorvall LYNX 4000 Superspeed Centrifuge, Thermo Scientific, Massachusetts, USA) at 12 000 x g, 30 min, at 4ºC. The resulting bacterial pellet was washed once with the same volume of sterile phosphate buffer saline (PBS x1; VWR, Radnor, Pennsylvania, USA) and resuspended in 200 mL physiological saline solution (NaCl; 0.9% w/v). In turn, for *B. animalis* BB-12®, one subsequent culturing step at the same growth conditions was performed, with a final incubation volume of 200 mL of MRS with 0.05% cysteine with 1% (v/v) of cell inoculation. B. *animalis* BB-12® cells were collected by centrifugation (Hettich Universal 320R Centrifuge, Andreas Hettich GmbH & Co. KH, Tuttlingen, Germany) at 3850 x g, 10 min, at 4ºC. The resulting bacterial pellet was washed once with the same volume of sterile PBS and then resuspended in 200 mL of NaCl 0.9% (w/v) solution. Both saline bacterial suspensions were stored at 4ºC for a maximum period of 24 h until chocolate preparation.

2.2. Chocolate production and storage

On the day of chocolate production, 70 mL of bacterial suspensions were centrifuged at 3850 x g, 10 min, at 4ºC, and the supernatants were discarded, while the bacterial pellets were maintained for incorporation. After this, 70 g of chocolate nuggets (containing 33.6% (Recipe Nº 823), 54.5% (Recipe Nº 811) or 70.5% (Recipe Nº 70-30-38) cocoa content) purchased from Callebaut (Lebbeke-Wieze, Belgium) were melted in a chocolate melting pot (Meilleur du Chef, Bassussary, France) until a temperature of 50ºC was reached. Next, the temperature was lowered by mixing and dripping the chocolate preparation until a temperature of 37ºC (optimum temperature to guarantee cell viability), and at this moment, bacterial pellets were incorporated into the chocolate by mixing thoroughly. Afterward, the chocolate mixture was spread onto a polycarbonate mold with half-spherical wells. After filling the wells, the mold was tossed a few times to remove the air bubbles eventually present. The chocolates were left to solidify at room temperature (20 ± 2ºC) for 4 h. Finally, chocolates were removed from the mold, obtaining around 15 single-serving chocolates stored in aluminum-wrapped Petri dishes at room temperature (20 ± 2ºC) under aerobic conditions. Also, plain/control (without probiotics) chocolates (for each cocoa content tested) were prepared following the same previous steps except for the incorporation of bacterial pellets.

2.3. Viability assessment in the bacterial suspensions and the probiotic chocolates throughout aerobic storage

The number of colony-forming units (CFU) of *A. muciniphila* DSM 22959 and *B. animalis* subsp. *lactis* BB-12® were determined at different time points: 1) in the saline bacterial suspensions directly after their preparation; 2) in the same saline bacterial suspensions immediately before their centrifugation to obtain pellets for incorporation into chocolate (to account for any losses during the storage period); 3) in chocolate samples immediately after production (i.e., at day 0 of storage); and 4) in chocolate samples after 7, 14, 21 and 28 days of aerobic storage at room temperature (20 ± 2ºC). For conditions 1 and 2, bacterial suspensions were 10-fold diluted in PBS, and 10 µL of each dilution were plated, in triplicate, onto PYGM agar (1.5% (w/v) bacteriological agar, Biokar diagnostics, Beauvais Cedex, France) for *A. muciniphila* DSM 22959 and MRS with 0.05% (w/v) cysteine and 1.5% (w/v) agar for *B. animalis* subsp. *lactis* BB-12®. For conditions 3 and 4, two single-serving chocolate samples (technical duplicates) were weighed and dissolved in pre-warmed (at 37ºC) PBS (in a proportion of 1:9). From this

suspension, decimal dilutions were performed in PBS, and 10 µL of each dilution were plated, in triplicate, on the appropriate media. Next, agar plates were incubated at 37ºC under anaerobic conditions during 5-7 days for *A. muciniphila* DSM 22959 and 48 h for *B. animalis* subsp. *lactis* BB-12®. After incubation, CFU enumeration was performed, and the results were expressed as mean ± standard deviation CFU/mL for bacterial suspensions (conditions 1 and 2) and mean ± standard deviation CFU/g in probiotic chocolate samples (conditions 3 and 4).

2.4. Physicochemical characterization of the probiotic chocolate

2.4.1. Preparation of chocolate extracts

The chocolate extracts were prepared according to the method described by Silva et al. (2017) with minor modifications. Initially, 5 mL of the previously diluted chocolate samples (section 2.3) were defatted using 10 mL n-hexane 97% (Ibis Scientific, Las Vegas, USA). Next, this solution was homogenized by agitation, followed by sonication (Bath sonicator, Bandelin, Berlin, Germany) for 5 min and centrifugation at 2470 x g for 5 min at 20ºC (Hettich Universal 320R Centrifuge, Andreas Hettich GmbH & Co. KH, Tuttlingen, Germany). The supernatant was then removed with a Pasteur pipette. This procedure was performed twice, and the samples were left to air-dry the residual n-hexane for approximately 1 h.

Posteriorly, the extraction of the phenolic compounds was performed by adding ethanol at 80% (v/v). At this moment, 20 mL of absolute ethanol (VWR, Pennsylvania, USA) were added to the remaining pellet and homogenized by a sonication step of 10 min, followed by a centrifugation step at 3850 x g for 5 min at 20ºC (Hettich Universal 320R Centrifuge, Andreas Hettich GmbH & Co. KH, Tuttlingen, Germany). Next, the supernatant was collected in a new 50 mL tube, and the procedure was repeated by adding 8mL of absolute ethanol and 2 mL of deionized water. Then, a rotavapor (Buchi, Flawil, Switzerland) was used to concentrate the phenolic content of the samples. Each sample was exposed to the rotavapor for approximately 30 min with a bath temperature of 45ºC and a pressure of 100 atm. Finally, a final volume of 5 mL of the sample was obtained.

2.4.2. Determination of the total phenolic content

The total phenolic compounds were determined using the Folin–Ciocalteu colorimetric method following the protocol described by Singleton & Rossi (1965) and Coscueta et al. (2018), with slight modifications.

Firstly, a calibration curve of gallic acid (0.025-0.200 mg/mL) was prepared to allow the expression of the results as milligrams of gallic acid equivalents per milliliter of sample (mg GAE/mL).

The assay consists of adding 30 μL of each sample (or its necessary dilution), 100 μL of Folin-Ciocalteu solution (20% v/v), and 100 μL of anhydrous sodium carbonate solution (7.4% w/v) to each assigned well. The microplate, wrapped in aluminum paper, was incubated for 30 min at 25ºC in the dark. The resulting blue mixtures were read at 765 nm on the Multi-detection plate reader (Synergy H1, Vermont, USA) operated using the Gen5 software. The results were expressed as mg of gallic acid equivalent per g of chocolate (mg GAE/g).

2.4.3. 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) scavenging assay

The ABTS assay was performed in a 96-well microplate, as described by Gonçalves et al. (2009), with slight modifications.

Firstly, the concentration of the ABTS working solution was adjusted to an initial absorbance of 0.70 (± 0.02) at 734 nm. Then, the Trolox solution was prepared by weighing 0.0125 g of Trolox (Sigma-Aldrich, Missouri, USA) and dissolving in 1 mL of methanol (Fischer Chemical, Massachussets, USA), completing the 50 mL volume with deionized water.

For the assay, 20 μL of Trolox or sample or solvent and 180 μL of ABTS working solution were added to each well. The 96-well microplate was incubated for 5 min at 30ºC, and the absorbance was measured at 734 nm with a Multi-detection plate reader (Synergy H1, Vermont, USA).

2.4.4. Angiotensin-I converting enzyme (ACE)-inhibitory activity assay

The ACE-inhibitory activity assay was performed in a 96-well black microplate, as described by Sentandreu & Toldra (2006), with minor modifications.

Firstly, 40 μL of ultrapure water or ACE working solution (42 mU/mL) were added to each correspondent well. Next, the final 80 μL volume was adjusted by adding ultrapure water to blank, control, or samples. Finally, the enzyme reaction was started by adding 160 μL of substrate solution (0.45 mM), and the mixture was incubated at 37ºC. The generated fluorescence was then measured at 30 min using a Multi-detection plate reader

(Synergy H1, Vermont, USA), with excitation and emission wavelengths at 350 and 420 nm, respectively.

2.4.5. α-Glucosidase inhibition assay

The antidiabetic potential was measured through the assessment of α-glucosidase inhibitory activity following the procedure described by Kwon et al. (2008), with some modifications. Firstly, 50 μL of samples were mixed with 100 μL of 0.1 M phosphate buffer (pH=6.9) containing α-glucosidase solution (1.0 U/mL) per each well of 96 wells microplate, and the mixture was incubated at 25ºC for 10 min. Afterward, each well was added to 50 μL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH=6.9). At this moment, the absorbance was read, and the reaction mixtures were incubated at 25ºC for 5 min for further absorbance readings using a Multi-detection plate reader (Synergy H1, Vermont, USA) at 405 nm.

For this assay, a negative control containing 50 μL of buffer solution in the place of the sample and positive control containing 50 μL of acarbose at a concentration of 10 mg/mL were used.

2.4.6. Sensory analysis – Triangular Test

For the sensory analysis, chocolate with 70.5% cocoa content incorporated with *A. muciniphila* DSM 22959 and a control chocolate were prepared according to section 2.2. In this context, a triangular test, targeting to determine whether the consumer could distinguish between a plain chocolate and the same chocolate matrix incorporating a probiotic strain, was performed. This studied involved 12 trained taste testers with ages ranging from 35-65 years old. Each tester performed two rounds of identification, in which three single-doses (whether probiotic or control chocolate) were served, and the participant was required to identify the corresponding chocolate in a form (Annex 6).

2.4.7. Aspect evaluation

To monitor the aspect of the different chocolates throughout the 28-day period of aerobic storage weekly pictures were taken of the single-serve doses, in duplicate.

2.4.8. Water activity (a_w), pH, and surface color determination

The chocolate pH values were assessed by dissolving single-serving chocolates in pre-warmed (at 37ºC) PBS in a proportion of 1:9 and analyzing the resulting suspension using a pH meter (Crison Instruments, Barcelona, Spain).

The same single-serving samples were used sequentially for the analysis of surface color and then a_w determination.

Thus, single-serving chocolates with homogeneous surfaces were selected to analyze the surface color using an appropriate colorimeter (Chroma meter CR 400, Konica Minolta, Osaka, Japan). This device provided the values of parameters "L", "a" and "b", which were used to calculate the whiteness index (WI), according to Lohman and Hartel's (1994) formula:

$$WI = 100 - [(100 - L)^2 + a^2 + b^2]^{0.5}$$

Afterward, single-serving chocolates were finely macerated using ceramic mortar and pestle and analyzed using a LabMaster-aw neo (novasina, Lachen, Switzerland) to measure a_w.

For all these analyses (a_w, pH, and surface color), two single-serving chocolates, representing technical duplicates, of each tested condition were used. These analyses performed at time points 1 and 28 days of aerobic storage.

2.4.9. Texture analysis

Chocolate hardness was determined using a Texture analyzer (TA. XT plus, Stable Micro Systems, Godalming, UK) with a load cell of 5 kg and a needle probe at control temperature of 20ºC.

2.4.10. *In vitro* simulation of the gastrointestinal tract

An *in vitro* simulation of the GIT as performed at the time point 1 day to test the protective power of the chocolate matrix on viable cell numbers of *B. animalis* BB-12® and *A. muciniphila* cells. As a control for this assay, *B. animalis* BB-12® and *A. muciniphila* free cells were stored in a NaCl 0.9% (w/v) solution at 25ºC. For each replica, either 0.5 mL of free cells in NaCl or 0.5 g of previously macerated chocolate were exposed to the simulated GIT conditions according to the protocol developed by Brodkorb et al. (2019), with slight modifications.

Firstly, the electrolyte solutions for each phase of the gastrointestinal tract passage – Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF), and Simulated Intestinal Fluid (SIF) – were prepared according to Table 2.1.

Table 2. 1 Composition of electrolyte solutions for each phase of the GIT protocol (Brodkorb et al., 2019)

Simulated solutions		**SSF**	**SGF**	**SIF**
		pH (7)	pH (3)	pH (7)
Chemical compound	Stock concentration	mmol/L	mmol/L	mmol/L
KCl	37.3 g/L	15.1	6.9	6.8
KH_2PO_4	68 g/L	3.7	0.9	0.8
$NaHCO_3$	84 g/L	13.6	25	85
$MgCl_2$ $6H_2O$	30.5 g/L	0.15	0.1	0.33
$(NH_4)_2CO_3$	48 g/L	0.06	0.5	-
NaCl	117 g/L	-	47.2	38.4
$CaCl_2$	44.1 g/L	1.5	0.15	0.6

For each experiment, the enzyme solutions – pepsin, pancreatin, and bile salts – were freshly prepared. The human GIT peristaltic movements and temperature were replicated using an orbital shaker incubator (Wiggen Hauser, Berlin, Germany) at 37ºC and 120 rpm.

Starting with the oral phase (OP), 1 mL of SSF was added to each sample. Next, in the esophagus-stomach step (gastric phase), samples were exposed to 2 mL of SGF containing pepsin (2000 U/mL – from porcine gastric mucosa; Sigma-Aldrich, Missouri, USA), and the pH was adjusted to 3 by using 6 M HCl. Next, the samples were exposed for 120 min to the gastric phase conditions. Lastly, 4 mL of SIF for the intestinal phase, containing pancreatin (100 U/mL – from porcine pancreas, Sigma-Aldrich, Missouri, USA) and bile salts (Fluka, North Carolina, USA), were added to the samples, and the pH was adjusted to 7, resorting to either 6 M HCl or 6 M NaOH. Samples were then incubated in the orbital shaker incubator for 180 min.

Samples were collected at the end of each phase, i.e., after 2 h of the gastric phase and after 3 h of the intestinal phase. At each defined sampling point, the total viable cell numbers of *B. animalis* BB-12® and of *A. muciniphila* DSM 22959 (free cells and incorporated in chocolate) were determined by plating, in triplicate, on MRS with 0.05% (w/v) cysteine agar and PYGM agar plates, respectively. This protocol was performed

under aerobic conditions, while the agar plates were incubated under anaerobic conditions.

2.5.Statistical analysis

Experiments were performed once and two technical replicates were carried out, in which plating was performed in triplicate. Data were analyzed using a statistical package for the social science 17.0 software (SPSS; Chicago, Illinois, USA). Parametric tests were carried out if the data followed a normal distribution according to Shapiro-Wilk test (Normality test). With this, for the statistical analysis of bioactivities, One-Way analysis of variance (ANOVA) test was used to evaluate differences between different conditions and cocoa contents, while for the comparison between two timepoints t-Student test for paired samples was applied. Additionally, for the statistical comparison between control and probiotic chocolate, t-Student for independent samples test was performed. Statistical differences were considered significant at P values < 0.05.

3. Results and Discussion

The present thesis has as its main goal the development of a chocolate matrix that acts as a probiotic delivery system for *Bifidobacterium animalis* subsp. *lactis* BB-12® and *Akkermansia muciniphila*. For this, the chocolate matrix was tested regarding several parameters, namely protective power in terms of probiotic viability during production and over a 28-day storage period and during GIT simulation. Furthermore, physicochemical characteristics including a_w, pH, color, hardness, and total phenolic compounds content were assessed. Besides that, important bioactivities including antioxidant, antidiabetic, and antihypertensive properties were tested. Both *B. animalis* BB-12® – classified as a traditional probiotic – and *A. muciniphila* – a proposed Next Generation Probiotic – have been the target of investigation regarding human health benefits and possible delivery systems. However, no study has yet proposed the incorporation of the free cells of these two probiotics in a chocolate matrix. Therefore, the following sections describe and analyse the results obtained regarding the several parameters assessed.

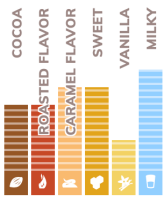
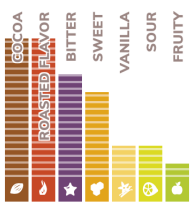
3.1. Viability of probiotics before, during chocolate preparation, and throughout aerobic storage

Consumer requirement for healthier foods with enhanced organoleptic properties has encouraged the food industry to develop functional foods combining bioactive ingredients that may supplement essential nutrition (food supplement) or exert a pharmacological activity (nutraceuticals). Specifically, chocolate could be used as a suitable carrier to deliver probiotic microorganisms, mainly due to its high acceptability by consumers ensuring improved compliance (Faccinetto-Beltrán, Gómez-Fernández, Santacruz, et al., 2021). One of the most important factors to consider when incorporating probiotics into food, such as chocolate matrices, is their viability throughout food production and subsequent storage (Silva et al., 2017; Succi et al., 2017). Although there is no unanimity regarding the minimum number of probiotic microorganisms needed to confer health-beneficial effects, it is widely accepted that a minimum level of 10^6 to 10^7 CFU/mL or CFU/g of viable probiotic cells must be present in the final product at the time of consumption (Kechagia et al., 2013; Pupa et al., 2021; D. M. Rasika et al., 2021).

The present work used chocolate nuggets of different cocoa content, obtained from the Belgian chocolate producer Callebaut, to produce the different chocolates plain (control) or added with a probiotic strain. The three different cocoa content percentages selected

for the study were 33.6% (w/w) (Recipe Nº 823), 54.5% (w/w) (Recipe Nº 811), and 70.5% (w/w) (Recipe Nº 70-30-38), the latter two being categorized as dark chocolate. The following table (Table 3.1) depicts the various characteristics, such as fluidity, composition, flavor profile, and nutritional declaration of the three used chocolate nugget types. It is important to note how the used ingredients profiles vary according to the cocoa content. For instance, chocolate with 33.6% and 54.5% cocoa content contain cocoa butter, contrary to the 70.5% cocoa content chocolate, which is produced with fat-reduced cocoa. This information will be important later when comparing and discussing bacterial cell viability, which may be influenced by various factors.

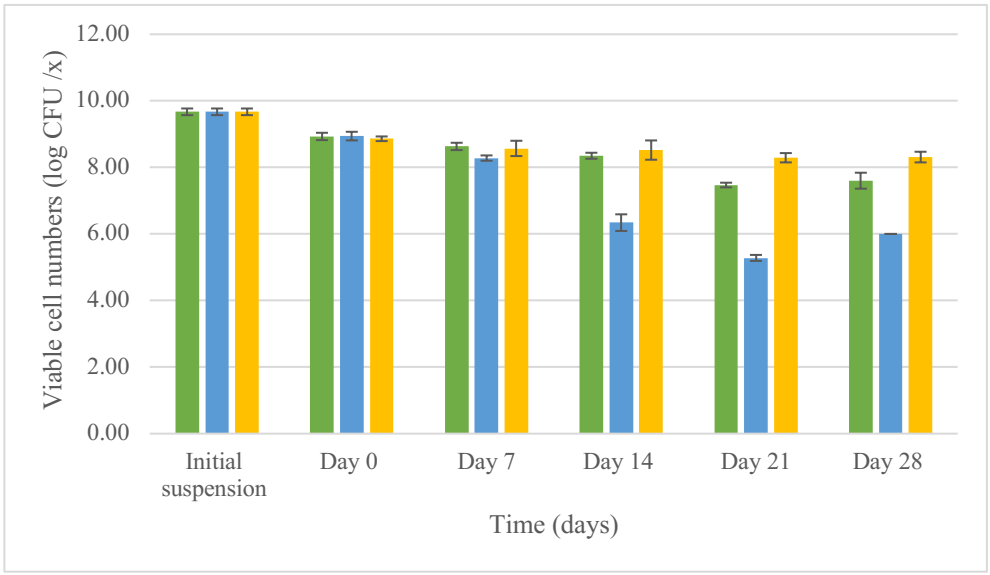
Table 3. 1 Characteristics of the different chocolate pellets used in chocolate production (Callebaut, 2008)

	Cocoa content [% (w/w)]		
	33.6%	**54.5%**	**70.5%**
Fluidity	Medium	Medium	Low
Ingredients	Cocoa mass Sugar Cocoa butter Whole milk powder Emulsifier: soya lecithin Natural vanilla flavoring	Cocoa mass Sugar Cocoa butter Emulsifier: soya lecithin Natural vanilla flavoring May contain: Milk	Cocoa mass Sugar Fat-reduced cocoa powder Emulsifier: soya lecithin Natural vanilla flavoring w: Milk
Composition			
Minimum Cocoa Percentage	33.6%	54.5%	70.5%
Minimum Milk Percentage	20.8%	0%	0%
Fat Percentage	36.2%	36.6%	38.9%
Flavor Profile			
Nutrition declaration per 100g			
Energy	550 kcal/ 2.303kJ	563 kcal/ 2.357kJ	539 kcal/ 2.255kJ
Fat	36.6g	36.2g	38.9g
of which saturates	22g	22g	23g
Carbohydrates	46g	51g	31g
of which sugars	43g	50g	26g
Protein	5.1g	7.0g	8.8g
Salt	0.01g	0.21g	0.02g

As it can be observed in Figures 3.1 a) and 3.1b), the bacterial suspensions of *A. muciniphila* DSM 22959 and *B. animalis* subsp. *lactis* BB-12®, used for the incorporation in chocolate, presented viable cell numbers between 10^9 and 10^{10} CFU/mL. A slight increase in viability of both probiotic bacteria was verified between the time points after preparation and before incorporation, demonstrating the importance of allowing the cells to recover after being exposed to a stress condition – in this case, centrifugation. The

centrifugation conditions were adjusted and adapted according to each bacterium used. In the case of *A. muciniphila*, whose cells are relatively small (0.6 – 1.0µm (Gómez-Gallego et al., 2016)), higher centrifugation speeds (10.000 – 12.000 x g (Barbosa et al., 2022)) were required to obtain the highest cell yield possible. However, after incorporation in chocolate, a decrease in cell viability was observed for both bacteria and in all three different cocoa percentages (33.6%, 54.5%, and 70.5%). Less than one log cycle reduction was reported for *B. animalis* subsp. *lactis* BB-12® whereas for *A. muciniphila* DSM 22959 the viability reduction was higher than 1 log cycle, particularly in the 70.5% cocoa percentage chocolate, where a reduction greater than 2 log CFU was observed.

(a)



(b)

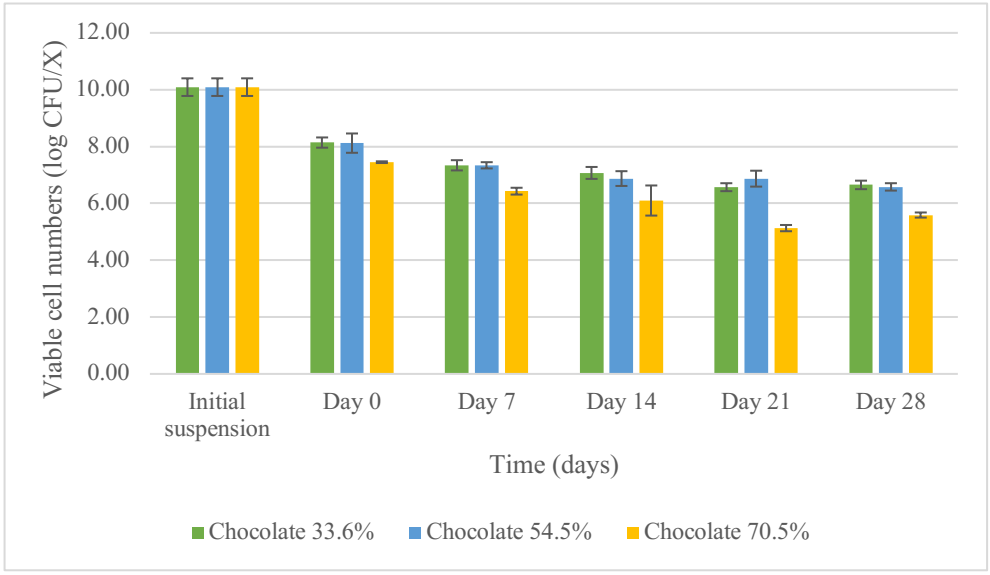


Figure 3. 1 Viable cell number of *B. animalis* subsp. *lactis* BB-12® (a) and *A. muciniphila* DSM 22959 (b) in initial suspension (log CFU/ml) and their evaluation in different cocoa content chocolates (log CFU/g) during aerobic storage at room temperature for 28 days.

Concerning the aerobic storage of *B. animalis* subsp. *lactis* BB-12®, a gradual decrease was observed throughout the 28 days in all chocolate matrices. However, the highest survival was verified for the chocolate with the highest cocoa content (mean value of 0.55 log CFU/g reduction, when comparing day 0 versus day 28), followed by the chocolate matrix with 33.6 % of cocoa (mean value of 1.30 log CFU/g reduction, when comparing day 0 versus day 28) (Annex 1). On the opposite, the chocolate matrix containing 54.5% cocoa content presented the lowest protective power (mean value around 2.95 log CFU/g loss, when comparing day 0 versus day 28) (Annex 1). Despite the viability reduction, all chocolate matrices allowed the survival of *B. animalis* subsp. *lactis* BB-12® at levels acceptable for a probiotic product, which should have a minimum cell concentration between $10^6 – 10^7$ CFU/mL or CFU/g to exert their beneficial health effect (Imlay, 2013; Kechagia et al., 2013; D. M. Rasika et al., 2021. In the literature, it has been reported that certain bioactive compounds present in chocolate, such as flavonoids, may act either as a prebiotic (supporting/ stimulating the viability of the probiotic bacteria) or as an antimicrobial agent (de Llano et al., 2017; Foong et al., 2013; Klindt-Toldam et al., 2016; Nazzaro et al., 2020). In this alignment, these results suggest that the chocolate with 70.5% cocoa content could act as a prebiotic substrate for *B. animalis* subsp. *lactis* BB-12®. Ongoing research has been focusing on the effect of cocoa polyphenols on human health; more specifically, studies aim to answer how these bioactive molecules shape the human gut microbiota (Sorrenti et al., 2020). Recent studies have, therefore, concluded that total dietary polyphenols are responsible for shaping the microbiota by 1) exerting prebiotic effects on beneficial bacteria and 2) having selective antimicrobial action against gastrointestinal pathogens (Kumar Singh et al., 2019; Tzounis et al., 2011). A 2011 *in vitro* study showed how the exposure of gut bacteria such as *Bifidobacterium* and *Lactobacillus* to water-insoluble cocoa fractions of commercially available cocoa powder leads to an increased bacterial growth rate, which consequently results in increased antioxidant action by the cocoa polyphenols (Fogliano et al., 2011). Additionally, a 2011 human study on the consumption of dairy-based cocoa beverages indicated the increased growth rate of *Bifidobacterium* with the consumption of polyphenols. This increase leads to several health outcomes, such as decreased cholesterol concentrations, and reduced plasma triacylglycerol concentrations (Tzounis et al., 2011).

Moreover, cocoa polyphenols have antimicrobial activity against Gram-positive and Gram-negative bacteria; however, a 2017 study has demonstrated how after “Dutching”,

an alkalization process used in the manufacture of several chocolate, the antibacterial power is greater against Gram-negative bacteria (Todorovic et al., 2017).

In the case of *B. animalis* BB-12®, our results suggest that polyphenols only exert beneficial prebiotic properties, when present at higher quantities, in a more accessible form, which is the case of the chocolate matrix with a cocoa content of 70.5%, which contains 80.17 ± 1.81mg gallic acid equivalent (GAE) per g of chocolate on day 1 (Figure 3.3). Otherwise, at lower concentrations – such as 7.27 ± 0.92 mg GAE/g of chocolate (33.6% cocoa content) or 24.71 ± 1.21mg GAE/g of chocolate (54.5% cocoa content) – the cocoa phenolic compounds are not sufficient to exert such prebiotic effect, and the matrix environment (including water activity, sugar content and casein-phenolic interactions) may lead to decreased cell viability in the chocolate matrices. Indeed, the chocolate matrix with 54.5% cocoa content has the highest sugar content (Table 3.1); a higher sugar content may increase osmotic pressure, which may lead to a reduction in viable cell numbers.

The obtained results were not expected, as predictably cocoa polyphenols would have the same effect on the probiotic independently of the concentration. Therefore, possible explanations were explored and hypothesized. As depicted in Table 3.1, the three used matrices have different composition and do not only vary in cocoa content. In other words, chocolate's ingredients diverge according to the desired cocoa content and contain, thus, variable trace elements. Hereby, a possible explanation for the growth inhibition in the matrix with 54.5% cocoa content could be the presence of trace elements, which could have antibacterial properties. Additionally, it could be hypothesized how the trace elements by themselves could not have inhibitory activity but could promote interactions with the phenolic compounds or other elements, which could cause the inhibition of bacterial growth. Secondly, it is important to note that chocolate matrices 33.6% and 54.5% are produced with cocoa butter, while the chocolate matrix with 70.5% cocoa content is manufactured with fat-reduced cocoa powder. Therefore, it is speculated that the different chocolates contain diverse phenolic compounds. Hereby, the phenolic compounds in cocoa matrices 33.6% and 54.5% act as antimicrobial agents, while the phenolic content in 70.5% cocoa content chocolate has prebiotic properties. To prove this hypothesis, spectrophotometric or chromatographic techniques should be performed to identify the phenolic compound's nature (Khoddami et al., 2013).

Concerning cell viability, it is also important to note that the chocolate matrix with lower cocoa content (33.6%) unavoidably has a higher milk content (20.8%), which consequently has a protective power over microbial cells (Table 3.1). Typically, dairy matrices are considered the most effective probiotic delivery systems due to their high nutrients availability, adequate pH, and high fat content, which protect cell viability (Neffe-Skocińska et al., 2018; C. S. Ranadheera et al., 2018; R. D. C. S. Ranadheera et al., 2010). Therefore, it was expected that the chocolate matrix containing milk would also be a suitable carrier for *B. animalis* BB-12®.

During the 28-day storage period, one of the monitored characteristics was the chocolate bonbons weight. From table 3.2, it is possible to calculate the overall average for the chocolate bonbon's weight, which is 2.42 g. Considering the above discussed results, it is possible to conclude how a single dose bonbon, weighing around 2.5 g of chocolate, contains the minimum required cell concentration for a probiotic product to exert health-beneficial effects on human health. As mentioned above (section 1.2) cocoa polyphenols and flavonoids are reported to exert positive effects on human health conditions such as depression and anxiety, obesity, cognitive functions, and cardiovascular diseases. Although this matter is still controversial, in 2012 EFSA has established a recommended intake of high-flavanol dark chocolate of 10 g per day to prevent high blood pressure and angina pectoris ("Scientific Opinion on the Substantiation of a Health Claim Related to Cocoa Flavanols and Maintenance of Normal Endothelium-dependent Vasodilation Pursuant to Article 13(5) of Regulation (EC) No 1924/2006," 2012). Considering the average weight of each single-dose chocolate, the consumer can enjoy a maximum of four single-doses daily.

Table 3. 2 *B. animalis* subsp. *lactis* BB-12® incorporated chocolate bonbons' weight during aerobic storage

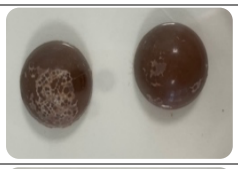
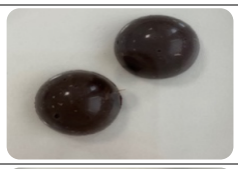
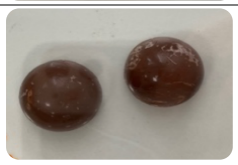
	Cocoa content [% (w/w)]		
	33.6%	**54.5%**	**70.5%**
Day 0	2.52 ± 0.11g	2.52 ± 0.02g	2.46 ± 0.04g
Day 7	2.51 ± 0.03g	2.46 ± 0.04g	2.31 ± 0.12g
Day 14	2.53 ± 0.03g	2.53 ± 0.10g	2.41 ± 0.00g
Day 21	2.55 ± 0.06g	2.50 ± 0.14g	2.42 ± 0.05g
Day 28	2.31 ± 0.05g	1.91 ± 0.13g	2.39 ± 0.02g
Overall average	2.48 ± 0.10g	2.38 ± 0.26g	2.39 ± 0.07

Another property, which is important to monitor during storage is the overall quality aspect of the chocolate. Throughout the 28-day period of aerobic storage, weekly pictures of the single-serve doses of the three tested chocolate matrices were taken and can be seen in Table 3.3. When comparing the three different tested conditions, it is possible to conclude that the color varies according to the cocoa content of the chocolate. The chocolate with the lowest cocoa content has the lightest color among the three chocolate types, while the chocolate with the highest cocoa content has the darkest shade. It is important to note that, for the naked eye, the color difference between the cocoa percentage 54.5% and 70.5% is practically undetectable, as both chocolates are already categorized as dark chocolate.

When assessing the chocolate overall quality aspect throughout a storage period, two important phenomena might occur, namely sugar and/or fat bloom. Chocolate's physical behavior is mainly determined by the cocoa butter, thus the requirement of strict tempering and storage temperatures, to assure the best physical properties. When chocolate is handled unproperly, undesirable properties might resurface, namely a bloom, which might be classified either as sugar or fat bloom (Kinta & Hatta, 2012). A bloom occurs when the fine structure created by either fat or sugar crystals is disrupted and therefore the chocolate surface becomes non-uniform. Sugar bloom is mainly caused by water factors, such as high humidity or contact with water, while fat bloom is triggered by high temperatures and/or dissolution of the fat in oil. Comparing both type of crystals, fat crystals are more sensitive to environmental factors and therefore less stable. Consequently, under ordinary conditions, fat bloom is more likely to occur compared to sugar bloom (Kinta & Hatta, 2012).

By analyzing the figures in Table 3.3, it is possible to observe that the three chocolate matrices at time point 0 have a shining surface, which is gradually lost throughout the 28-day storage period. The phenomenon of fat bloom is particularly evident in the chocolate matrices of 33.6% and 70.5% cocoa content. To determine whether it is, or not fat bloom illustrative pictures of both bloom occurrences were used as a comparison base (Annex 7). Although fat bloom might alter organoleptic properties such as aspect and flavor, it does not affect cell viability, as both affected matrices had the highest cell survival rate (Figure 3.1a)). Particularly, homemade chocolate is affected by this type of occurrences as temperature control is less rigorous compared to that performed in an industry environment. Therefore, homemade chocolate has a shorter shelf life of approximately 30 days when stored at room temperature.

Table 3. 3 Evolution of the overall quality aspect of chocolates incorporated with *B. animalis* subsp. *lactis* BB-12® throughout 28 days of aerobic storage

	Cocoa Percentage [% (w/w)]		
	33.6%	54.5%	70.5%
Day 0			
Day 7			
Day 14			
Day 21			
Day 28			

During the aerobic storage of the chocolates incorporated with *A. muciniphila* DSM 22959, the largest decrease in cell viability was observed in the first 14 days, with a reduction in cell viable numbers ranging between 1-2 logs CFU/g depending on the chocolate matrix type (Figure 3.1b); Annex 2). After this period, cell viability reduced but in lesser magnitude. The chocolate matrices with 33.6% and 54.5% cocoa content exhibited a similar protective effect on *A. muciniphila* viability, allowing survival around the minimum number required of 10^6 CFU/g after 28 days of aerobic storage. Only the matrix with 70.5% cocoa content did not achieve the minimum required levels for probiotic products. Interestingly, in the case of *A. muciniphila*, the phenolic compounds do not act as prebiotic substances, as cell viability decreases with the increase of cocoa content. As stated above, cocoa polyphenols have greater antimicrobial activity against Gram-negative bacteria, particularly, after the chocolate is subjected to the alkalinization process, which consequently increases the antibacterial power (Todorovic et al., 2017). Considering that *A. muciniphila* is a Gram-negative bacterium, the observed behavior of decreases in cell viability with increased cocoa polyphenols content was expected. In the

literature, the incorporation of NGP, namely *A. muciniphila*, into chocolate matrices remains poorly explored. In fact, Marcial-Coba and colleagues (2019) were pioneers when they used 70% dark chocolate as a carrier for freeze-dried microencapsulated *A. muciniphila* in a xanthan/gellan gum matrix and evaluated bacterial survival during anaerobic storage and during *in vitro* gastric transit. Embedding in dark chocolate conferred increased protection to the encapsulated *A. muciniphila*, resulting in viable cell numbers higher than 10^6 CFU/g after 60 days of anaerobic storage (at 4ºC and 15ºC) (Marcial-Coba et al., 2019). However, the anaerobic storage conditions applied by Marcial-Coba *et al.* do not correspond to a feasible storage modality (more expensive and unsuitable for a household context). Moreover, using an encapsulation procedure before the incorporation in chocolate increases the production costs of this functional chocolate, which becomes less attractive to the industry.

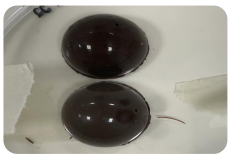
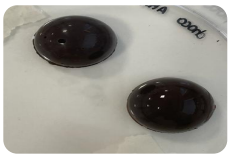
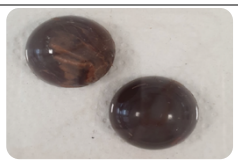
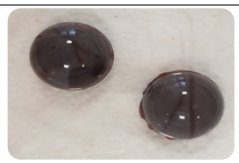
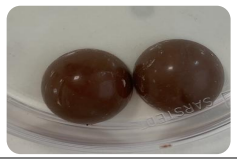
During the 28-day storage period, the single-dose chocolates incorporated with *A. muciniphila* were weighed weekly. Based on table 3.4, it is possible to calculate the average for the chocolate bonbon's weight, which is around 2.40g, being very similar to that found for the chocolate bonbon's weight incorporating *B. animalis* BB-12® (2.42g). As mentioned before, a single dose bonbon comprises the minimum required viable cell numbers for a probiotic product to exert health-beneficial effects on human health. Hereby, the same conclusion as before can be withdrawn, which means the consumer can enjoy a maximum of four single-doses daily of the 54.5% probiotic chocolate to achieve the recommended dark chocolate intake to prevent high blood pressure and angina pectoris according to EFSA ("Scientific Opinion on the Substantiation of a Health Claim Related to Cocoa Flavanols and Maintenance of Normal Endothelium-dependent Vasodilation Pursuant to Article 13(5) of Regulation (EC) No 1924/2006," 2012).

Table 3. 4 *A. muciniphila* DSM 22959 incorporated chocolate bonbons' weight during aerobic storage

	Cocoa content [% (w/w)]		
	33.6%	**54.5%**	**70.5%**
Day 0	2.34 ± 0.04g	2.41 ± 0.08g	2.42 ± 0.15g
Day 7	2.35 ± 0.07g	2.46 ± 0.07g	2.37 ± 0.04g
Day 14	2.39 ± 0.03g	2.48 ± 0.11g	2.43 ± 0.11g
Day 21	2.39 ± 0.01g	2.46 ± 0.04g	2.38 ± 0.01g
Day 28	2.42 ± 0.04g	2.46 ± 0.06g	2.37 ± 0.23g
Overall average	2.38 ± 0.03g	2.45 ± 0.03g	2.39 ± 0.03g

Also, in this case the overall quality aspect of the chocolate was monitored during the 28-day aerobic storage period. Based on Table 3.5, the same conclusions as before can be withdrawn. As mentioned earlier, the chocolates' color becomes darker with the increase in cocoa content.

Table 3. 5 Evaluation of the overall quality aspect of chocolates incorporated with *A. muciniphila* throughout 28 days of aerobic storage

	Cocoa Percentage [% (w/w)]		
	33.6%	**54.5%**	**70.5%**
Day 0			
Day 7			
Day 14			
Day 21			
Day 28			

By evaluating the photographs in Table 3.5, it is possible to observe that only the dark chocolate matrices at time point day 0, upon production, have a shining surface, which is progressively lost throughout the storage period. The phenomenon of fat bloom is particularly evident in all three chocolates, starting as early as upon production (at timepoint day 0) for the 33.6% cocoa content chocolate matrix. This phenomenon is mainly due to unmanageable temperature oscillations, which leads to alteration in the fine crystal structure. Once again, it is important to note that, although fat bloom might alter organoleptic properties such as overall quality aspect and flavor, it does not affect cell viability, as both affected matrices had the highest cell survival rate (Figure 3.1b)).

3.2. Chemical and biological characterization of chocolates

Chocolate, in particular dark chocolate, has the potential to confer benefits to human health due to the presence of a grand panoply of bioactive compounds (Araujo et al., 2016). This is mainly due to the more than 300 chemical compounds present in the cocoa bean itself. Hereby, these compounds include bioactive molecules such as polyphenols (flavonoids and non-flavonoids); methylxanthines (theobromine and caffeine) and minerals (magnesium, iron, and zinc) (Petyaev & Bashmakov, 2017). The physicochemical characterization of chocolates included the analysis of several parameters such as pH, total phenolic compounds, and antioxidant, antidiabetic, and antihypertensive activities.

3.2.1. Evaluation of pH

As it can be seen in Figure 3.2, the pH values ranged between 6.09 and 6.80. Furthermore, a decrease in pH values with an increase in cocoa content can be observed in both control and probiotic chocolates. Natural cocoa powder is characterized as a low acidic product with pH values ranging from 5.3 to 5.8, due to the presence of organic acids, such as acetic and lactic acids, which are a result of the fermentation of sugars. However, cocoa powder undergoes an alkalization process (also known as “Dutching”), which results in a darker color (brownish red to black) of the cocoa mass and consequently raises the pH values to 6.8 to 8.1. In a chocolate product, the pH is responsible for the reduction of the microbial activity, therefore avoiding the possible growth of food pathogens such as *Salmonella*. Lower pH values of a chocolate matrix have led to increased sour and astringent taste. Contrary, higher pH values result in an intensified dark color and consequently affect flavor and taste of the cocoa mass (Aprotosoaie et al., 2016; Valverde García et al., 2020).

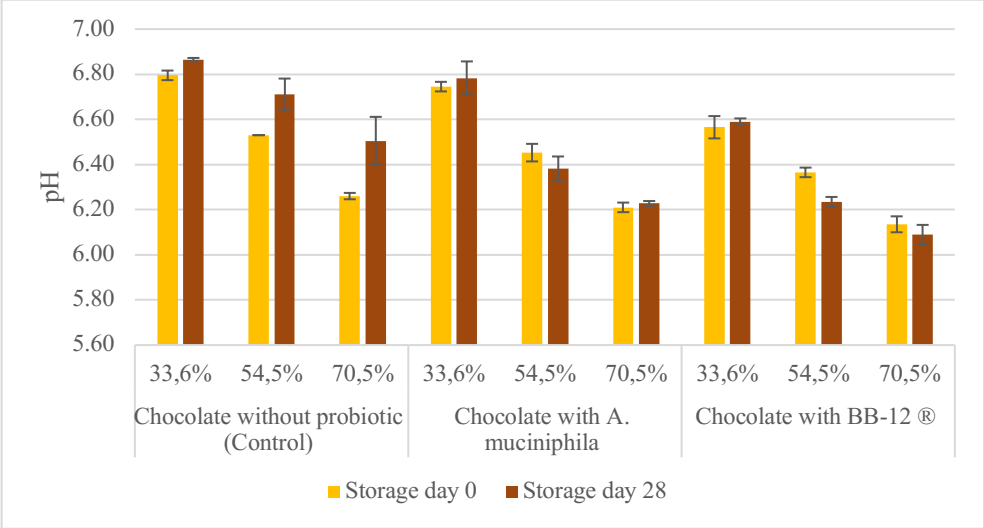


Figure 3. 2 Evaluation of pH values between 0 and 28 days of aerobic storage in all three tested chocolate matrices (33.6%, 54.5% and 70.5% cocoa contents) for all analyzed conditions: control chocolate, chocolate incorporating *A. muciniphila* DSM 22959 and chocolate incorporating *B. animalis* subsp. *lactis* BB-12®.

Importantly, adding probiotics to chocolate did not change the pH values significantly compared to those of the plain chocolate control ($p > 0.05$). Also, the pH values in all chocolates (with/ without probiotics) remained highly stable throughout the 28 days of aerobic storage at room temperature, only with slight oscillations, i.e. lower than 1.0 in the pH scale (Annex 3). These findings are in accordance with previous studies involving functional chocolates incorporating probiotic lactobacilli and bifidobacteria strains and indicate that the added probiotic strains either had a slowed down metabolism or were metabolically inactive throughout storage. This characteristic is of greater importance for preserving food products throughout their storage period (Silva et al., 2017b; Succi et al., 2017b).

3.2.2. Determination of total phenolic compounds and bioactivities

A clear pattern can be identified concerning chocolates' total phenolic compounds and antioxidant activity; specifically, with the increase of cocoa content in a chocolate matrix, an increment in total phenolic compounds and antioxidant activity was observed (Figures 3.3 and 3.4, respectively). Indeed, total phenolic compounds ranged between 5.65-7.27, 24.23-25.04, and 43.27-80.17 mg GAE/g for chocolates with cocoa percentages of 33.6%, 54.5%, and 70.5%, respectively (Annex 4). In turn, antioxidant activity varied between 52146.43-81702.45, 205909.00-232146.81, and 349916.87-416219.14 mg of Trolox equivalent per g of sample for each chocolate 33.6%, 54.5%, and 70.5%, respectively

(Annex 4). Regarding this matter, Mikolajczak and Tanska verified that cocoa mass content in chocolate bars was strongly correlated with phenolic compounds and antioxidant capacity (Mikołajczak & Tańska, 2021). Also, Kemsawasd et al. demonstrated that white, milk, and dark chocolates (containing 0%, 10%, and 50% of cocoa, respectively) presented values of total polyphenols and antioxidant activity in ascending magnitude (Kemsawasd et al., 2016).

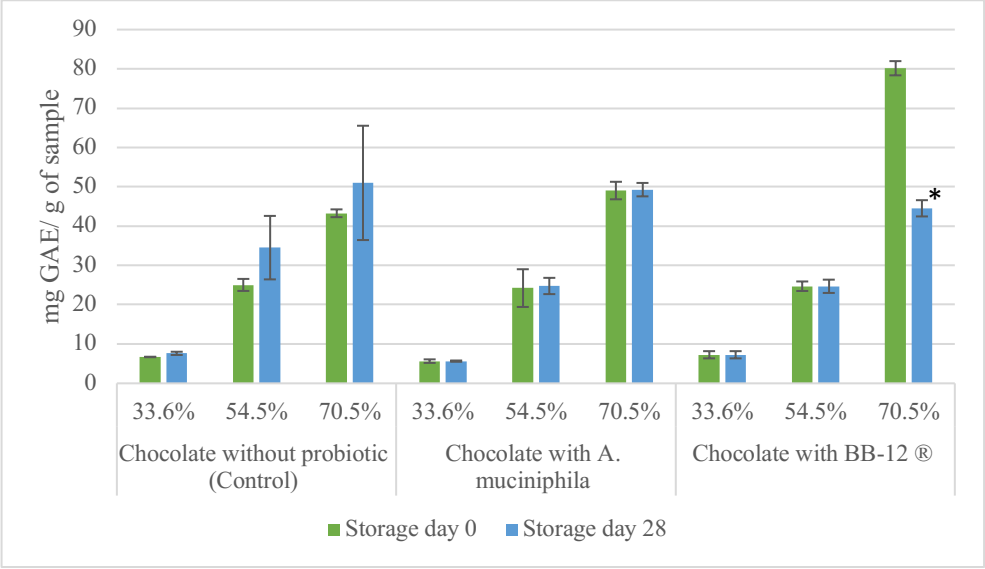


Figure 3. 3 Total phenolic compounds content between 0 and 28 days of aerobic storage in all three tested chocolate matrices (33.6%, 54.5% and 70.5% cocoa contents) for all analyzed conditions: control chocolate, chocolate incorporating *A. muciniphila* DSM 22959 and chocolate incorporating *B. animalis* subsp. *lactis* BB-12®. Statistical differences between storage day 0 and day 28 are marked with *.

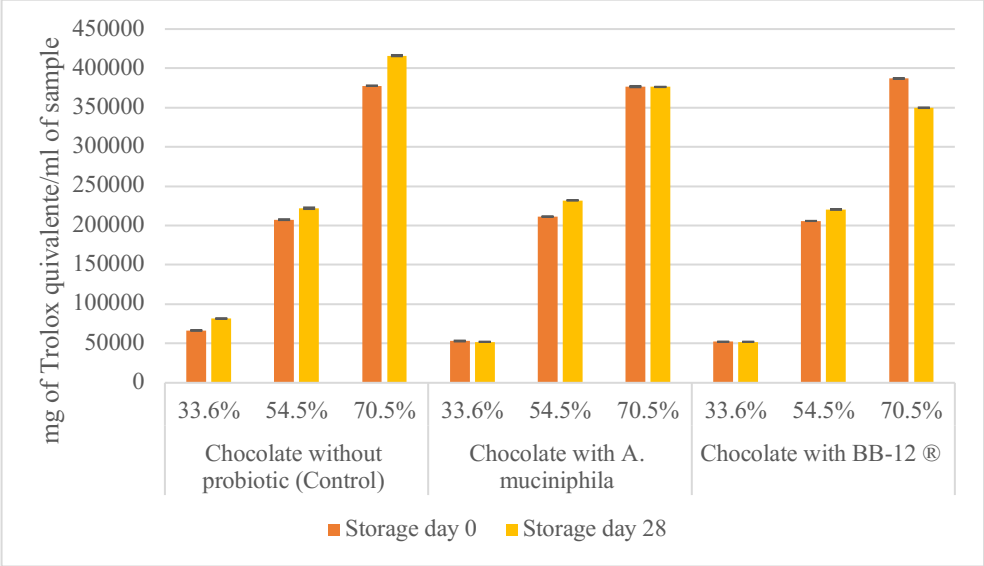


Figure 3. 4 Antioxidant activity between 0 and 28 days of aerobic storage in all three tested chocolate matrices (33.6%, 54.5% and 70.5% cocoa contents) for all analyzed conditions: control chocolate, chocolate incorporating *A. muciniphila* DSM 22959 and chocolate incorporating *B. animalis* subsp. *lactis* BB-12®.

Moreover, within the same cocoa content, the plain chocolate (control) and both probiotic-incorporated chocolates showed similar values for the total phenolic content and the antioxidant activity ($p > 0.05$) and mostly did not present statistical differences throughout 28-day aerobic storage at room temperature ($p > 0.05$, Figure 3.3 and Figure 3.4, respectively). Similarly, Silva *et al.* (2017) demonstrated semisweet chocolate incorporated with *Lactobacillus acidophilus* LA3 and *Bifidobacterium animalis* subsp. *lactis* BLC1 did not present statistical differences in the total content of phenolic compounds when compared with the control chocolate. However, the 70.5% cocoa content chocolate incorporated with the probiotic *B. animalis* BB-12® appears as the exception, since the values for total phenolic compounds vary greatly between day 0 and day 28 ($p < 0.05$). Despite this difference the same effect is not observed for the antioxidant activity. On day 28 of aerobic storage the total phenolic compounds content was found to be half of the initial value and a possible explanation for this event could be the consumption of the phenolic compounds by the bacteria. Consequently, these results imply a beneficial prebiotic property by the cocoa polyphenols as previously described.

Regarding the total phenolic compounds of *A. muciniphila* incorporated chocolates, it is possible to observe that the values for all three cocoa percentages (33.6%, 54.5% and 70.5%) do not vary greatly during the storage time ($p > 0.05$). Thus, it is possible to conclude that for *A. muciniphila*, the phenolic compounds have antibacterial activity. In

sum, the increase of phenolic content leads to an increased antimicrobial activity in the chocolate matrix.

Although for some conditions, the total phenolic compounds contents vary significantly between timepoint 0 days and timepoint 28 days, the antioxidant activity is constant ($p > 0.05$), indicating that the bioactivity of the phenolic compounds is not drastically affected by the loss of phenolic compounds throughout the storage period. As such, it could be hypothesized that the antioxidant activity is more related with the phenolic compounds that remain stable throughout the storage period.

Concerning antidiabetic and antihypertensive activities, an increasing trend in these biological properties was observed with the increase of cocoa content of the chocolate matrix (Figure 3.5 and Figure 3.6).

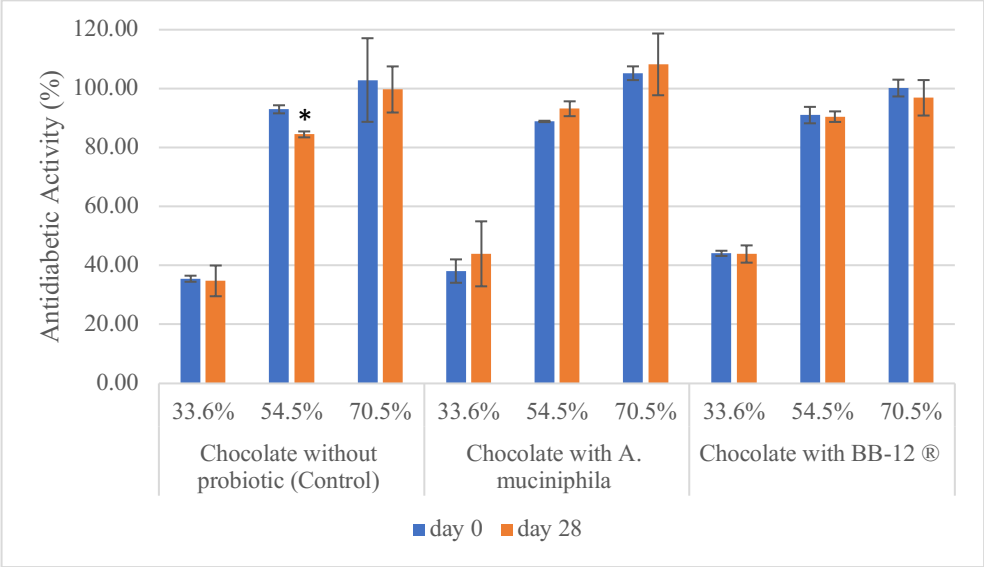


Figure 3. 5 Antidiabetic activity between 0 and 28 days of aerobic storage in all three tested chocolate matrices (33.6%, 54.5% and 70.5% cocoa contents) for all analyzed conditions: control chocolate, chocolate incorporating *A. muciniphila* DSM 22959 and chocolate incorporating *B. animalis* subsp. *lactis* BB-12®. Statistical differences between storage day 0 and day 28 are marked with *.

Particularly, for antidiabetic activity, the incorporated chocolates with *A. muciniphila* all three cocoa percentages 33.5%, 54.5% and 70.5% do not present statistically different values during aerobic storage ($p > 0.05$) (Figure 3.5; Annex 5). For the control statistical differences throughout storage are observed in the chocolate with 54.5% cocoa content ($p < 0.05$), while the values of antidiabetic activity are kept constant throughout the 28-day storage period for cocoa percentages 33.6% and 70.5% (Figure 3.5). These observations show that in the control chocolate storage leads to a loss of activity while in

the experimental probiotic-containing chocolates an increase in activity is observed post probiotic addition. In particular, results reveal the possibility of higher antidiabetic activity due to the presence of *A. muciniphila*, which has been broadly studied regarding its antidiabetic properties. However, for the chocolate incorporated with *B. animalis* BB-12® no statistical differences are observed during the storage period for the three tested cocoa percentages (Figure 3.5). Nonetheless, it was perceived that within the same cocoa content, the control and both probiotics incorporated into chocolates showed similar values and did not present statistical differences throughout the 28-day aerobic storage at room temperature ($p > 0.05$). This indicates that predominantly the addition of probiotic bacteria does not impact the antidiabetic activity either positively or negatively.

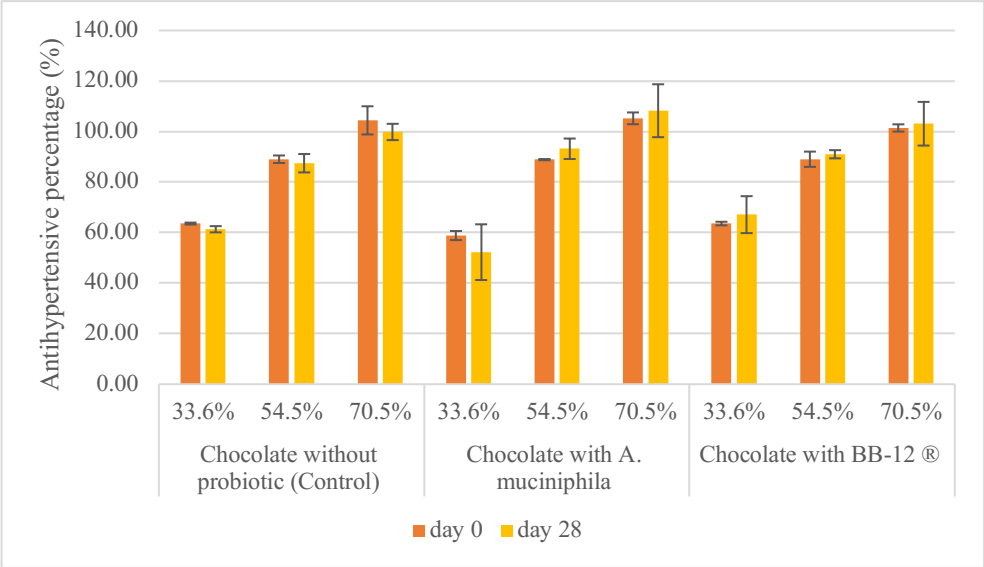


Figure 3. 6 Antihypertensive activity between 0 and 28 days of aerobic storage in all three tested chocolate matrices (33.6%, 54.5% and 70.5% cocoa contents) for all analyzed conditions: control chocolate, chocolate incorporating *A. muciniphila* DSM 22959 and chocolate incorporating *B. animalis* subsp. *lactis* BB-12®.

Regarding the antihypertensive activity (Figure 3.6), all three percentages of cocoa content varied significantly from each other for the three tested conditions (control, *B. animalis* BB-12® and *A. muciniphila*) ($p < 0.05$). In other words, the higher the cocoa content, the higher the antihypertensive activity (Annex 5; Figure 3.6). Moreover, it was observed that within the same cocoa content, the control and both probiotics incorporated into chocolates showed similar values and did not present statistical differences throughout the 28-day aerobic storage period at room temperature ($p > 0.05$). Therefore,

it is important to note how the addition of probiotics does not affect either positively or negatively the antihypertensive properties of the three tested chocolate matrices.

Specifically, dark chocolate has antihypertensive properties, due to its ability to lower the occurrence of high blood pressure by enhancing the circulation of nitric oxide (Samanta et al., 2022). Also, dark chocolate grants antidiabetic properties to the consumer, as it increases insulin sensitivity, resulting therefore in reduced insulin resistance (Samanta et al., 2022). However, the antihypertensive and antidiabetic activities in functional chocolates incorporating probiotics remain unexplored, and therefore, no further data are available. It is important to note that *in vivo* studies point out that *A. muciniphila* has the potential to reduce body mass gain and plasma TNF-α levels, increase the number of goblet cells and mucin secretion, and restore gut barrier function (Deng et al., 2022). All these effects contribute to the amelioration of diabetes related syndromes. The performed assay on antidiabetic activity does not target insulin sensibility, instead it determines the inhibition of α-glucosidase. Therefore, a correlation between the incorporation *A. muciniphila* in a chocolate matrix and increase on antidiabetic activity cannot be performed, as two different parameters are being evaluated. Although, the addition of *A. muciniphila* did not affect the antidiabetic activity in the chocolate matrices, the incorporation of the probiotic in chocolate might still be valuable regarding this property, as the bacterium might act against diabetes in the human system after consumption. To prove this hypothesis, an *in vivo* study targeting the antidiabetic properties of chocolate incorporated with *A. muciniphila* must be performed.

3.3. Sensory Analysis

Flavor, aspect, smell, and texture are decision-based criteria for the consumer when purchasing a chocolate (Lemarcq et al., 2022). Sensory analysis emerges as a useful tool, which compromises a wide range of tests, to determine consumer's perception of the food in question (Drake, 2007). Hereby, the sensory quality is the ultimate measure for a product's quality and consequent success. In this context, a triangular test aiming to determine whether the consumer could distinguish between a plain chocolate and the same chocolate matrix incorporating a probiotic strain (Annex 6). This triangular test was performed using chocolate matrix with 70.5% cocoa content and *A. muciniphila* DSM 22959 as probiotic strain.

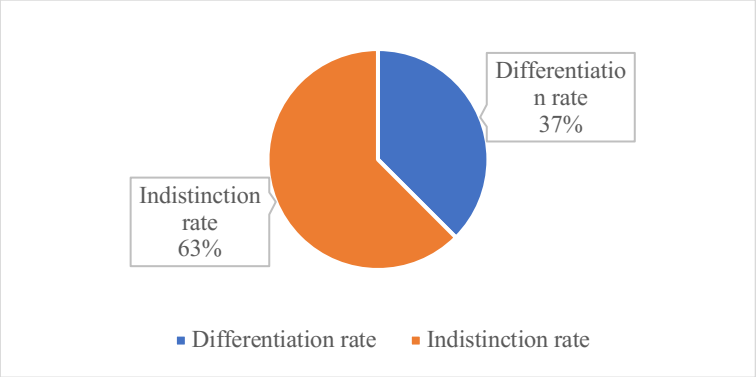


Figure 3. 7 Percentage of differentiation and indistinction of the Triangular Test performed for the chocolate matrix 70.5% cocoa content incorporated with *A. muciniphila* DSM 22959

The test was performed with a total of 12 tasters, resulting in an indistinction rate of 63%, meaning that, on average, 7 of the 12 samplers were not able to distinguish between the incorporated and standard chocolates. The taste testers, who were able to differentiate both chocolates, pointed out to differences in the chocolates' texture. The difference in textures could occur during the preparation phase, when the probiotic is added to the chocolate. The addition of the probiotic results in a quicker cooling process of the chocolate mass, which consequently leaves less time to mechanically remove the air trapped inside the chocolate single-dose. This results in a bubblier chocolate interior due to the existing air bubbles. Although the test was only performed for *A. muciniphila*, the same results are expected for *B. animalis* BB-12®, as both probiotics are incorporated using the same strategy.

Nevertheless, although the addition of probiotics may lead to differences in the organoleptic properties, the added benefits of a probiotic incorporated chocolate could compensate these minor alterations and the consumer would, hereby, opt for this functional food instead of the standard available ones.

3.4. Detailed characterization of the best performing chocolate matrices for incorporation of each of the probiotic strains

3.4.1. Characterization of *B. animalis* BB-12® incorporated in chocolate with 70.5% of cocoa content

After determining the most appropriate chocolate matrix for the classic probiotic *B. animalis* subsp *lactis* BB-12®, the next step is based on the characterization of the chosen matrix in terms of survival during GIT passage, pH, water activity, texture, and whiteness index.

A new batch of bonbons was prepared, either with or without bacterial incorporation, and the previously determined parameters were assessed once again, to ensure that the same range of values would be obtained. As it can be seen in Figure 3.8, the bacterial suspensions of *B. animalis* subsp. *lactis* BB-12®, used for the incorporation in chocolate, had an initial cell concentration of 10^9 CFU/mL.

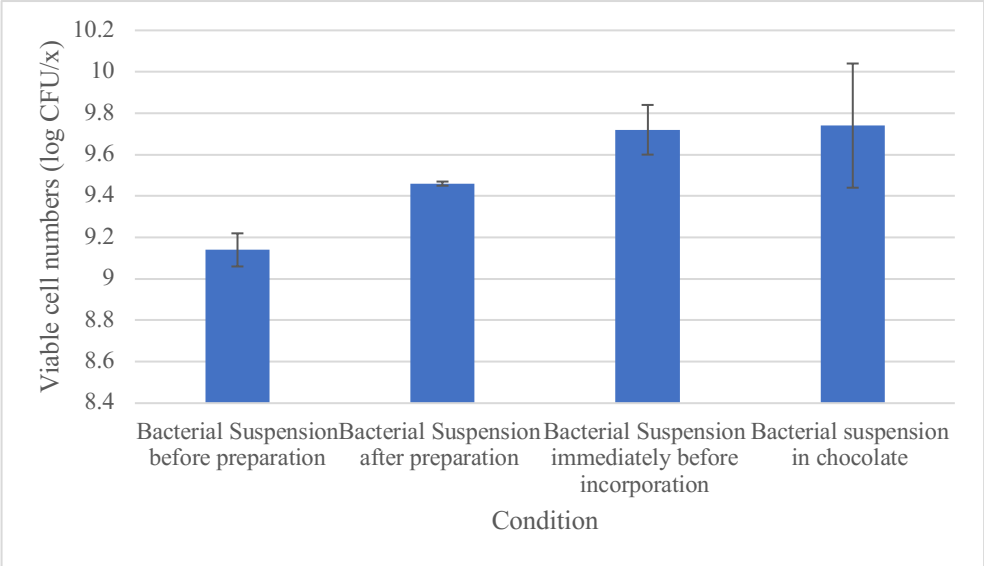


Figure 3. 8 Viable cell number of *B. animalis* subsp. *lactis* BB-12® in bacterial suspension before and after preparation (log CFU/ml), before incorporation (log CFU/ml) and when incorporated in the 70.5% cocoa content chocolate (log CFU/g)

Specifically, a slight increase in viability of the probiotic bacteria was verified between the time points after preparation and before incorporation, demonstrating the importance of allowing the cells to recover after being exposed to a stress condition. Contrary to the behavior observed previously (section 3.1), after incorporation in chocolate, no decrease in cell viability was observed for *B. animalis* BB-12®, with no statistical differences between timepoint immediately before incorporation and after incorporation (around log 9.7 CFU/g). Although the cell viability values are lower compared to the ones obtained in section 3.1., the resulting cell concentration complies to the minimum required levels for a probiotic product (10^6 – 10^7 CFU/g or CFU/mL) (Imlay, 2013; Kechagia et al., 2013).

Several parameters are used to assess chocolate's physiochemical properties. For this work, the following parameters were determined and analyzed: overall quality aspect, weight, water activity, pH, whiteness index and texture – including firmness (work of penetration) and hardness (maximum force) (Table 3.6).

Table 3. 6 Physico-chemical properties of the incorporated *B. animalis* subsp. *lactis* BB-12® chocolate with 70.5% cocoa content

		Chocolate with 70.5% (w/w) cocoa content	
		Control	With *B. animalis* BB-12®
Overall quality aspect			
Weight		2.28 ± 0.05 g	2.29 ± 0.06 g
Water activity		0.36 ± 0.01	0.62 ± 0.01
pH		6.20 ± 0.16	6.40 ± 0.04
Whiteness Index		22.0 ± 0.51%	22.1 ± 0.53%
Texture			
	Firmness (Work of Penetration)	9824.68 ± 86.27 N	14513.92 ± 207.22 N
	Hardness (Maximum Force)	1.38 ± 0.04 N	2.31 ± 0.19 N

All the parameters were determined for single-serve dose chocolates incorporated with *B. animalis* BB-12® and for control plain chocolates (without bacteria).

Starting with the overall quality aspect evaluation, it is possible to note that the plain chocolate (control) is shinier compared with the probiotic one. Additionally, the incorporated chocolate presents an uneven surface covered with small holes. As previously explained in section 3.1. and 3.3., the incorporation of a probiotic in a chocolate matrix led to a more rapid decrease of temperature when preparing the chocolate. Furthermore, in the same section, it was also described how chocolate's physical behavior is mainly determined by the cocoa butter, thus requiring strict tempering and storage temperatures, to assure the best physical properties. Hereby, the rapid temperature reduction led to 1) a less successful air removal step during chocolate preparation and 2) to undesired oscillations in temperature, which lead unavoidably to the phenomenon of fat bloom.

Furthermore, when comparing the whiteness index (WI) values of both incorporated and plain chocolates, it is possible to conclude that no statistically significant differences between both indexes can be observed ($p > 0.05$). The WI is the most used color parameter to assess storage parameters for chocolate (Quevedo et al., 2013). Chocolates affected by

the appearance of fat bloom develop a whitish outer layer on the surface, which can be measured and interrelated with the WI. Although for the human eye the whitish outer layer in the chocolate incorporated with *B. animalis* BB-12® is clear, in terms of whiteness index the differences do not present any statistical importance. In the work of Silva *et al* (2017) similar results were presented, in which the control and probiotic chocolate obtained similar values at timepoint 0 regarding WI. However, it is important to note how WI is expected to increase throughout aerobic storage, according to Silva et al.

Texture is characterized as a combination of the physical structure of the material and the mechanical and surface properties of said material (Andrae-Nightingale et al., 2009). In this case, texture was analyzed by determining two parameters, namely firmness (work of penetration) and hardness (maximum force). Hardness characterizes the physical rigidity of the chocolate and is highly linked to the sensory perception of the consumer. In turn, work of penetration depicts the work that is calculated by the area under the force curve and is correlated with the maximum force for penetration (Faccinetto-Beltrán, Gómez-Fernández, Orozco-Sánchez, et al., 2021b). Work of penetration is a measure often used as an index of firmness or hardness, being regularly applied to confectionary products. According to Table 3.6, firmness and hardness are higher for the chocolate containing the probiotic strain, indicating that the addition of *B. animalis* BB-12® could contribute to the chocolate's hardness. However, in their work, Facinetto-Beltran (2021) and Succi (2017) did not obtain similar results, as regarding hardness and work of penetration the values were not statistically different between control and probiotic chocolate. According to Shah et al, chocolate hardness is determined by the fat crystal structure, thus the more stable the structure, the harder the chocolate (Shah et al., 2010). Therefore, it is possible to hypothesize that, although the appearance of the whitish outer layer in the probiotic chocolate indicates the occurrence of fat bloom (disruption of the crystal structure), the rapid temperature decrease during chocolate preparation leads to a more rigid structure of all present crystals.

Concerning the pH values, the same conclusion as in section 3.2. can be withdrawn. In other words, the addition of the probiotic strain to chocolate did not change the pH values significantly compared to the control values. ($p > 0.05$). These results are in agreement with previous studies concerning functional chocolates, indicating that the present probiotic strains were metabolically inactive, which is of extreme importance for preserving food products throughout the storage period (Silva et al., 2017b; Succi et al., 2017b).

Lastly, concerning water activity, the values between control and probiotic chocolate vary greatly. The probiotic chocolate has double the water activity compared to the control (Table 3.6), which is most likely due to the addition of the probiotic in liquid form. During the chocolate preparation, the probiotic is added suspended in a sodium-chloride solution. Although the volume of the probiotic suspension compromises less than 5% of the total volume, it is enough to double the water activity. It is important to note that the minimum a_w to support a microorganism's growth is 0.60, although most bacteria require in average a a_w of 0.87 (Beuchat et al., 2013). Although the a_w value for probiotic chocolate is above the minimum a_w required for microorganisms to grow, the risk of bacterial contamination is low, as most require higher levels, being therefore the increased water activity of little concern.

For a probiotic product to be introduced into the industry with claims of health benefits, the probiotic cell concentration in said product must have a minimum of 10^6-10^7 CFU/g or CFU/mL as previously mentioned (Binda et al., 2020; Hur et al., 2011). In this context, the probiotic viable cell numbers in a food product should preferably be of 10^8-10^9 CFU/g or CFU/mL, before ingestion, to guarantee that when the probiotic cells reach the colon their numbers vary between 10^6-10^7 CFU/g or CFU/mL (Binda et al., 2020; Hur et al., 2011; Nazzaro et al., 2009). Hereby, the aim is for the beneficial action to occur in the intestine and therefore the probiotic cells must survive the harsh conditions of the human GIT. To understand the probiotic cells' behavior when exposed to the human GIT, *in vitro* digestions models are used. These models allow the simulation of the gastrointestinal conditions and consequently enable the study of cell behavior, structural changes, digestibility, and probiotic cell release from the food matrix (Jacobsen et al., 2020). As it can be seen in Figure 3.8, the chocolate with 70.5% cocoa content incorporated with the probiotic *B. animalis* BB-12® had an initial cell concentration of 10^8 CFU/g, following, the recommended viable cell numbers for a probiotic product before digestion.

After being submitted to the acidic condition of the stomach, the cell concentration seemed to increase (Figure 3.9). This increase in cell viability after exposure to the gastric phase conditions is not expected. A possible explanation for this might be that the chocolate when exposed to the peristaltic movements of the GIT releases the probiotic cells more efficiently compared to the mechanic method used for the enumeration of cells in the non-digested chocolate. After exposure to the intestinal conditions for three hours, cell viability accounts for a 0.5 log CFU/g loss, indicating that the chocolate matrix does protect the *B. animalis* BB-12® cells from the conditions of the GIT. For instance, Succi

(2017), who worked with probiotic strains such as *Lactobacillus rhamnosus* GG, *Lactobacillus casei* DG, *Lactobacillus paracasei* F19 and *Lactobacillus reuteri*, obtained similar results, concluding that the chocolate matrix is a potential carrier for probiotics, due to its protective power over the gastrointestinal stress (Succi et al., 2017a).

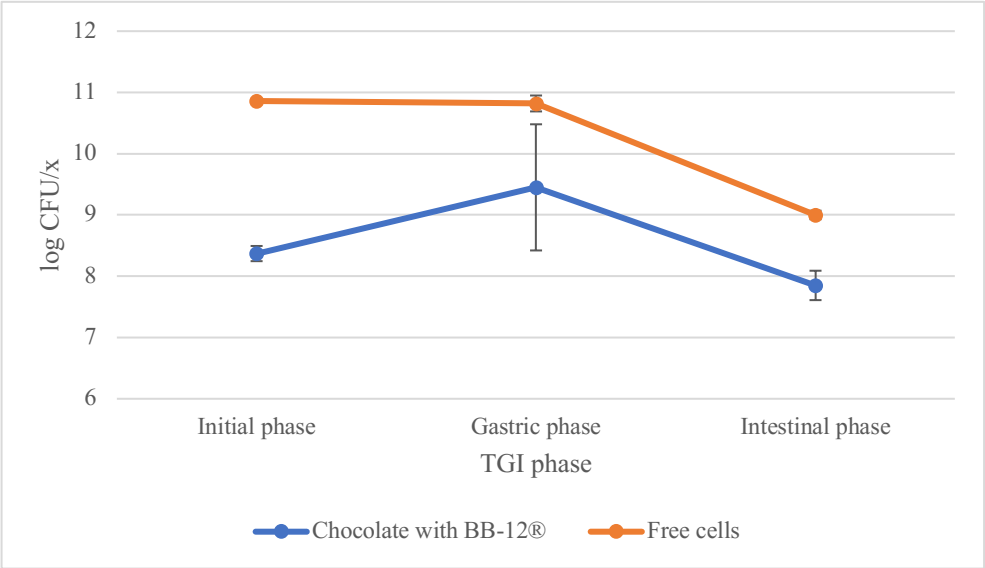


Figure 3. 9 Evolution of viable cell numbers of *B. animalis* subsp. *lactis* BB-12® in free form (log CFU/ml) or when incorporated in chocolate with 70.5% cocoa content (log CFU/g) during in *vitro* passage through gastrointestinal tract.

For comparative reasons, free cells of the probiotic *B. animalis* BB-12® were exposed to the GIT. In Figure 3.9 it is possible to observe that the initial viable cell numbers in free form were higher, when compared to the viable cell numbers in the chocolate; this is expected since the free cells were not exposed to the stress process of chocolate incorporation and, therefore, no cell loss was observed. Afterwards, a maintenance of free cells viability in gastric phase was found. Lastly, a high viability reduction for free cells (around 2 log cycles) was observed in the intestinal phase, being this reduction greater than that verified for chocolate incorporating *B. animalis* BB-12® (only around 0.5 log cycle). Thus, this finding suggests that chocolate used as a food delivery matrix ensures a higher protection for this probiotic strain throughout GIT passage.

3.4.2. Characterization of *A. muciniphila* incorporated in chocolate with 54.5% cocoa content

After the determination of the most appropriate chocolate matrix for *A. muciniphila* DSM 22959 – 54.5% cocoa content –, the same parameters as described in the previous section were characterized.

Figure 3.10 shows how the bacterial suspension of *A. muciniphila* DSM 22959, prepared for later incorporation in the chocolate with 54.5% cocoa content, had an initial cell concentration of 10^7 CFU/mL.

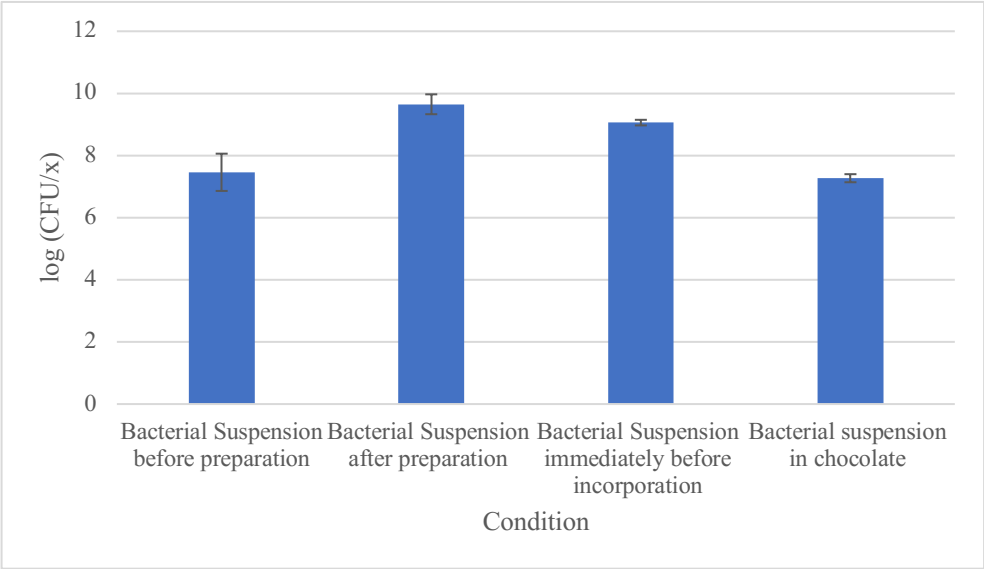


Figure 3. 10 Viable cell number of *A. muciniphila* DSM 22959 in bacterial suspension before and after preparation (log CFU/ml), before incorporation (log CFU/ml) and when incorporated in the 54.5% cocoa content chocolate (log CFU/g)

Throughout the various inoculum preparation steps, an increase in cell viability was observed between the time points after preparation and before incorporation. This phenomenon illustrates the importance of cell recovery, as mentioned above in section 3.1. and 3.4.1. As in section 3.1, after incorporation in chocolate, a decrease in cell viability was observed for *A. muciniphila* DSM 22959; nevertheless, the minimum required levels for a probiotic product (10^6 – 10^7 CFU/g or CFU/mL) are achieved (Imlay, 2013; Kechagia et al., 2013a).

As described in section 3.4.1, several parameters regarding chocolate's physiochemical properties were assessed and are presented in Table 3.7. Hereby, all properties were evaluated for single-serve dose chocolates incorporated with *A. muciniphila* DSM 22959 and for control chocolates (without bacteria).

Table 3. 7 Physico-chemical properties of the incorporated *A. muciniphila* DSM 22959 chocolate with 54.5% cocoa content.

		Chocolate with 54.5% (w/w) cocoa content	
		Control	With *A. muciniphila*
Overall quality aspect			
Weight		2.46 ± 0.01 g	2.41g ± 0.22 g
Water activity		0.50 ± 0.01	0.77 ± 0.01
pH		6.54 ± 0.03	6.38 ± 0.03
Whiteness Index		24.38 ± 1.47%	30.14±1.53%
Texture	**Firmness (Work of Penetration)**	6461.24 ± 2201.66 N	13664.28 ± 3582.19 N
	Hardness (Maximum Force)	0.90 ± 0.35 N	1.95 ± 0.61N

Regarding the overall quality aspect evaluation, it is visible that both plain (control) and probiotic-incorporated chocolate do not present the desired shine. Hereby, the occurrence of the phenomenon of fat bloom in both chocolates is, once again, highlighted. In sections 3.1. and 3.3. it was clarified how the incorporation of a probiotic in a chocolate matrix leads to a more rapid decrease of temperature throughout chocolate preparation. As mentioned earlier, chocolate's physical behavior is determined by the cocoa butter, which requires proper handling strict tempering and storage temperatures. Therefore, the rapid temperature reductions and the oscillations of room temperature may result inevitably in the appearance of fat bloom. Moreover, considering the whiteness index values of both probiotic-incorporated and plain chocolate (control), it is possible to conclude that these are statistically different ($p < 0.05$), indicating that the whitish outer layer of the incorporated chocolate is more prominent. Although the WI values are statistically different, results described in the literature depict contradictory outcomes since control and probiotic chocolate present similar values at timepoint 0 day regarding WI (Silva et al., 2017a). Additionally, in section 3.4.1., WI values between control and *B. animalis* BB-12® incorporated chocolates were similar, suggesting that the addition of probiotic does not lead to the fat bloom appearance. Consequently, it is possible to hypothesize that

the different WI values depicted in Table 3.7 are due to the preparation process and to possible temperature oscillations and not due to the addition of probiotic.

Concerning the values of the firmness and hardness, these are, once again, higher for the chocolate containing the probiotic bacteria, indicating that the addition of *A. muciniphila* could contribute to the chocolate's hardness ($p < 0.05$). The same conclusion was withdrawn in section 3.4.1. regarding the addition of *B. animalis* BB-12® to the chocolate matrix. Consequently, the following potential hypothesis was considered: despite the occurrence of fat bloom phenomenon, the swift temperature diminution during chocolate preparation results in a more rigid structure of all present crystals.

The obtained pH results demonstrate statistically different outcomes ($p < 0.05$). This is, adding probiotic strain to the chocolate does alter the pH values meaningfully, when compared to the control values. Contrary to the abovementioned statement for the incorporation of *B. animalis* BB-12®, the achieved results do not meet the outcomes of other studies concerning probiotic chocolates (Silva et al., 2017b; Succi et al., 2017b), suggesting, hereby, that the present probiotic strain might be metabolically active. Nevertheless, it might be hypothesized that the metabolic activity is reduced as the decrease in pH is only 0.2 in the pH scale.

Regarding water activity, the values of plain (control) and probiotic-incorporated chocolate are significantly different ($p < 0.05$), as, once again, the probiotic chocolate presents a higher water activity value in comparison to the control one (Table 3.7). As mentioned before, this might be due to the addition of the probiotic in liquid form, more concretely, the probiotic is added suspended in a NaCl solution 0.9% (w/v). However, it is important to note that the obtained values are not alarming, regarding pathogen growth, just as stated in section 3.4.1. Marcial-Coba *et al* determined the water activity for chocolates containing microencapsulated *A. muciniphila*, obtaining lower values, as the *A. muciniphila* cells were not in suspension but in the form of freeze-dried microcapsules.

Regarding the viability throughout simulated GIT conditions, as depicted in Figure 3.11, the chocolate with 54.5% cocoa content incorporating *A. muciniphila* DSM 22959 was present, in the initial phase, at 10^6 CFU/g. Taking into account the data of Figure 3.10, in which the chocolate incorporating *A. muciniphila* DSM 22959 shows a cell concentration of 10^7 CFU/g at timepoint 0, it is possible to observe a cell loss of almost 1 log cycle after a 24-hour aerobic storage. Although the initial cell viability was not as high as expected, it's important to analyze whether the cells survive the GIT stress conditions.

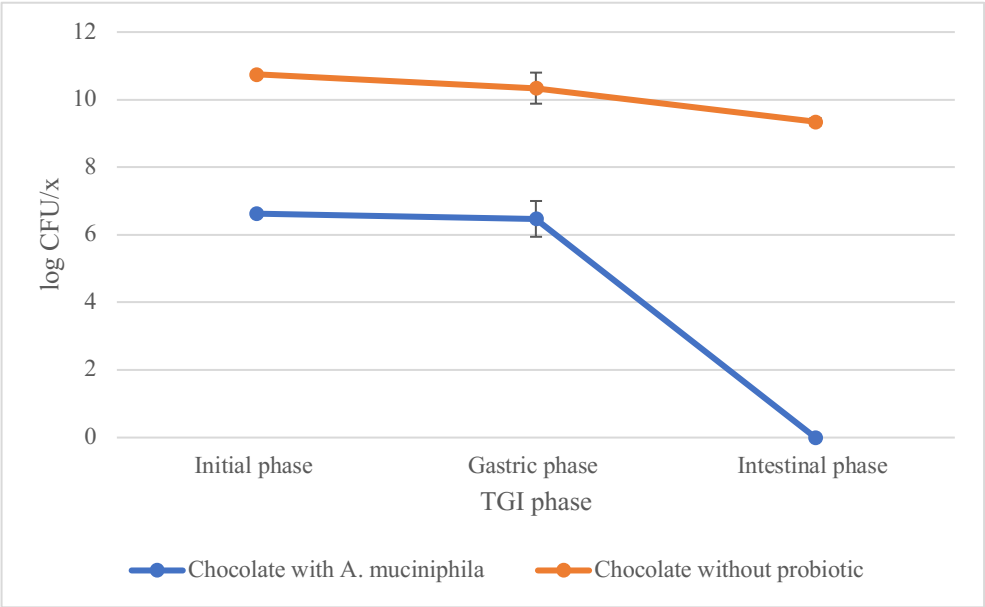


Figure 3. 11 Evolution of viable cell numbers of *A. muciniphila* DSM 22959 in free form (log CFU/ml) or when incorporated in chocolate with 54.5% cocoa content (log CFU/g) during in *vitro* passage through gastrointestinal tract.

After submission to the acidic condition of the stomach, viable cell numbers were maintained, with a minimal loss of less than 0.5 log CFU/g. In a previous work from Marcial-Coba et al, the researchers worked with microencapsulated *A. muciniphila* cells, which were later incorporated into chocolate. These researchers evaluated the behavior of said chocolate during the exposure to the oral and gastric phase, obtaining less promising results, as cell viability decreased by 1 log CFU/g (Marcial-Coba et al., 2019). Although the present work showed promising results after exposure to the gastric phase of the GIT, cell viability was completely lost after exposure to the intestinal conditions for three hours, with cell viability counts lower than the limit of detection of CFU enumeration technique. This result was not predicted, since, according to the literature, the chocolate matrix has a great protective power over the gastrointestinal stress (Possemiers et al., 2010; Succi et al., 2017a). The obtained results lead to the speculation of possible reasons for these values, as in the literature, no author has yet described the behavior of *A. muciniphila* free cells in chocolate when exposed to the full gastrointestinal tract. Therefore, the following hypothesis has been proposed: the chocolate matrix when exposed to the intestinal phase might form or release active compounds, which might have antibacterial activity against *A. muciniphila* cells, which are more sensitive compared to classical strains belonging to the *Bifidobacterium* or *Lactobacillus* genus.

For comparative reasons, free cells of *A. muciniphila* were exposed simultaneously to the GIT. In the Figure 3.11, *A. muciniphila* DSM 22959 free cells in initial phase of *in vitro* GIT assay showed a higher viability than its chocolate counterpart, therefore, presenting a similar trend to observed to *B. animalis* BB-12®. In fact, free cells' behavior is more promising, compared to the incorporated one, as cell viability only decreases less than 2 log CFU/g after exposure to the *in vitro* GIT passage. However, even though free cells can survive the GIT without increased protection after 24-hour of aerobic storage, our investigation group demonstrated that after long storage periods the added protection of a matrix ensures cell viability to the required levels. To overcome this limitation, protective strategies, such as lyophilization or microencapsulation of *A. muciniphila* cells prior to incorporation into chocolate, should be addressed in further studies (Almeida et al., 2022).

4. Conclusion

The present study was the first to evaluate the effect of three different chocolate matrices (with 33.6%; 54.5% or 70.5% cocoa content) on the viability of *B. animalis* subsp. *lactis* BB-12® and *A. muciniphila* DSM 22959 during aerobic storage at room temperature for 28 days. In fact, dark chocolates with high cocoa content namely 70.5% and 54.5%, seem to be a promising food carrier to deliver probiotic bacteria *B. animalis* BB-12® and *A. muciniphila* DSM 22959, respectively.

Indeed, a chocolate matrix containing 70.5% cocoa content allowed the survival of *B. animalis* BB-12® at viable cell number levels of at least 10^8 CFU/g, after a 28-day aerobic storage period at room temperature. Additionally, after an *in vitro* simulated GIT passage, this matrix ensured viable cell numbers of *B. animalis* BB-12® around 10^7 CFU/g, fulfilling the minimum required threshold of 10^6 – 10^7 CFU/g for a probiotic product. Besides that, the chocolate with 70.5% of cocoa content exhibited the highest quantity of phenolic compounds as well as the most representative antioxidant, antidiabetic, and antihypertensive bioactivities.

Regarding *A. muciniphila* DSM 22959, the most appropriate chocolate matrix was the one with 54.5% cocoa content, as cell viability was kept at 10^6 CFU/g, after a 28-day aerobic storage. It is important to highlight that the chocolate matrix with 33.6% cocoa content presented similar results concerning cell viability, however, due to the increased bioactivity of the 54.5% cocoa content chocolate matrix, this one was chosen as the most suitable functional vector, due to the greater functionality. Although, *A. muciniphila* was able to thrive during aerobic storage in the chocolate matrix, results regarding *in vitro* gastrointestinal simulation were disappointing, as the bacterium was not able to survive the harsh GIT conditions, when incorporated into chocolate. Hereby, it was suggested that during digestion antibacterial compounds might be formed or released, which harm the bacterial cell, consequently leading to its death. The path for further investigation arises, with possible solutions including the lyophilization or microencapsulation of *A. muciniphila* before its incorporation in chocolate.

When developing a food product, specific aspects regarding food safety and food quality must be assessed to ensure that the addition of the probiotic does not affect the final product. For this purpose, pH and water activity for both control and probiotic chocolates were determined. Results indicated that the pH values did not vary greatly, indicating that

the cells were metabolically inactive, which is of great importance for preserving food products throughout a storage period. Water activity values increased with the addition of the probiotic, although a_w levels did not reach alarming results. Consequently, the addition of these specific probiotic strains seemed to not affect the chocolate in terms of food safety and food quality.

Lastly, parameters regarding sensory quality of the chocolate, such as aspect and texture, were evaluated. For both probiotics, results were similar indicating that the addition of probiotics might alter the external surface of the chocolate and might increase chocolate's hardness. However, a sensory triangle test indicated that the consumer had difficulties in differentiating a plain chocolate (control) and a probiotic chocolate. However, though the addition of probiotics leads to alterations in the organoleptic properties of the chocolate matrix, the supplementary benefits of a probiotic chocolate could overweight these minor variations and the consumer would, consequently, select for this functional food instead of the standard available ones.

5. Future work

The results presented in this thesis provided valuable information concerning the incorporation of *B. animalis* subsp. *lactis* BB-12® and *A. muciniphila* DSM 22959 in chocolate matrices and presented insights regarding the bioactivity and the performance of the incorporated chocolates when subjected to simulated GIT conditions. Nevertheless, a few questions have been raised throughout this study, which should be addressed in future research work.

Firstly, *A. muciniphila* cells were unable to survive the GIT conditions when incorporated in a chocolate matrix (section 3.4.1). So far, no studies concerning the incorporation of *A. muciniphila* free cells in a chocolate matrix have been published, lacking therefore information concerning cell behavior during the GIT simulation. This lack of information raises questions regarding *A. muciniphila* survivability in the GIT. Primarily, it would be interesting to understand which compounds are formed or released during digestion in the intestinal phase and whether they have antibacterial effects on *A. muciniphila* cells. Secondly, an additional investigation line emerges should focus on encapsulation techniques, including spray-drying and microencapsulation by internal gelation. Adding a primary layer of protection to the probiotic cells could potentiate the protective power of the chocolate matrix and therefore assure the survival of *A. muciniphila* during GIT simulation. The same investigation line could be applied to *B. animalis* BB-12®, as encapsulation techniques guarantee protection over long storage periods.

Another potential extension of this work is based on the incorporation of co-cultures, composed of *B. animalis* BB-12® and *A. muciniphila* DSM 22959. Hence, several parameters such as proportion and most adequate matrix regarding cocoa content should be investigated and analyzed. The incorporation of co-cultures in the chocolate matrix could potentially increase the functionality of the probiotic products, as the two chosen strains target different human health conditions.

Finally, another possible investigation line could be the improvement of the chocolate matrix itself. In other words, the enhancement of the chocolate formulation could increase the potential of the functional product. Hereby, some of the components, for instance a fat or a carbohydrate ingredient, could be replaced by a healthier ingredient or by compounds with a higher bioactive potential.

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Annexes

Annex 1

Table 3. 8 *B. animalis* subsp. *lactis* BB-12® viability before, during chocolate preparation, and throughout aerobic storage

Time Point	CFU/X ± S.D.		
	Chocolate matrix – cocoa content [% (w/w)]		
	33.6%	**54.5%**	**70.5%**
Bacterial Suspension after preparation		$2.73 \pm 1.77 \times 10^9$	
Bacterial Suspension immediately before incorporation		$4.70 \pm 1.14 \times 10^9$	
Day 0	$8.73 \pm 2.14 \times 10^8$	$9.01 \pm 2.14 \times 10^8$	$7.33 \pm 1.22 \times 10^8$
Day 7	$4.73 \pm 1.04 \times 10^8$	$1.95 \pm 0.37 \times 10^8$	$4.20 \pm 2.14 \times 10^8$
Day 14	$2.27 \pm 0.43 \times 10^8$	$2.53 \pm 0.11 \times 10^6$	$3.98 \pm 2.36 \times 10^8$
Day 21	$2.98 \pm 0.46 \times 10^7$	$1.95 \pm 1.05 \times 10^6$	$2.02 \pm 0.58 \times 10^8$
Day 28	$4.43 \pm 2.23 \times 10^7$	$1.00 \pm 0.00 \times 10^6$	$2.13 \pm 0.64 \times 10^8$

Annex 2

Table 3. 9 *A. muciniphila* DSM 22959 viability before, during chocolate preparation, and throughout aerobic storage

Time Point	CFU/X ± S.D.		
	Chocolate matrix – cocoa content [% (w/w)]		
	33.6%	**54.5%**	**70.5%**
Bacterial Suspension after preparation	$1.20 \pm 0.81 \times 10^{10}$		
Bacterial Suspension immediately before incorporation	$1.48 \pm 0.92 \times 10^{10}$		
Day 0	$1.46 \pm 0.51 \times 10^{8}$	$1.90 \pm 1.28 \times 10^{8}$	$2.80 \pm 0.20 \times 10^{7}$
Day 7	$2.38 \pm 1.07 \times 10^{7}$	$2.27 \pm 0.58 \times 10^{7}$	$2.78 \pm 0.79 \times 10^{6}$
Day 14	$2.27 \pm 0.43 \times 10^{6}$	$2.53 \pm 0.11 \times 10^{6}$	$3.98 \pm 2.36 \times 10^{6}$
Day 21	$3.85 \pm 1.23 \times 10^{6}$	$3.85 \pm 5.61 \times 10^{6}$	$1.38 \pm 0.32 \times 10^{5}$
Day 28	$4.75 \pm 1.65 \times 10^{6}$	$3.92 \pm 1.15 \times 10^{6}$	$3.97 \pm 0.83 \times 10^{4}$

Annex 3

Table 3. 10 pH values of all tested conditions during 28-day storage

Condition		**Storage**			
Sample	**Cocoa Percentage [% (w/w)]**	**Day 0**		**Day 28**	
		Mean	**S.D.**	**Mean**	**S.D.**
Chocolate without probiotics (Control)	**33.6%**	6.80	0.02	6.87	0.01
	54.5%	6.53	0.00	6.71	0.07
	70.5%	6.26	0.01	6,51	0.11
Chocolate with *A. muciniphila* DSM 22959	**33.6%**	6.76	0.02	6.78	0.07
	54.5%	6.43	0.04	6.38	0.05
	70.5%	6.20	0.02	6.23	0,01
Chocolate with *B. animalis* subsp. *lactis* BB-12®	**33.6%**	6.57	0.05	6.59	0,01
	54.5%	6.37	0.02	6.24	0.02
	70.5%	6.14	0.04	6.09	0.04

Annex 4

Table 3. 11 Total phenolic compounds and antioxidant activity of control chocolate, chocolate incorporated with B. animalis subsp. lactis BB-12® and chocolate incorporated with *A. muciniphila* DSM 22959

Condition		**Storage**			
		Total Phenolic Compounds		**Antioxidant activity**	
Sample	**Cocoa Percentage [% (w/w)]**	**Day 0**	**Day 28**	**Day 0**	**Day 28**
		mg GAE/ g of chocolate ± S.D.		**mg of Trolox equivalent /g of chocolate ± S.D.**	
Chocolate without probiotics (Control)	**33.6%**	6.71 ± 0.06	7.63 ± 0.41	66616.47 ± 54.45	81702.45 ± 41.18
	54.5%	25.04 ± 1.53	34.52 ± 8.08	207573 ± 580.15	222077.03 ± 1016.02
	70.5%	43.27 ± 0.99	50.99 ± 14.55	377923.66 ± 0.99	416219.14 ± 807.98
Chocolate with *A. muciniphila* DSM 22959	**33.6%**	5.65 ± 0.45	5.65 ± 0.16	53155.04 ± 574.44	52144.38 ± 18.76
	54.5%	24.23 ± 4.80	24.77 ± 2.07	211416.20 ± 133.62	232146.81 ± 60.87
	70.5%	49.05 ± 2.23	49.28 ± 1.71	376785.50 ± 808.76	376398.50 ± 231.94
Chocolate with *B. animalis* subsp. *lactis* BB-12®	**33.6%**	7.27 ± 0.92	7.27 ± 0.92	52200.05 ± 102.59	52146 ± 104.28
	54.5%	24.71 ± 1.21	24.69 ± 1.69	205909.00 ± 124.59	220541.04 ± 199.52
	70.5%	80.17 ± 1.81	44.53 ± 2.07	387106.49 ± 348.01	349916.98 ± 390.99

Annex 5

Table 3. 12 Anti-diabetic and anti-hypertensive activity of control chocolate, chocolate incorporated with *B. animalis* subsp. *lactis* BB-12® and chocolate incorporated with *A. muciniphila* DSM 22959

Condition		**Storage**			
		Anti-diabetic Activity		**Anti-hypertensive Activity**	
Sample	**Cocoa Percentage [% (w/w)]**	**Day 0**	**Day 28**	**Day 0**	**Day 28**
		Inhibitory Activity % ± S.D.		**Inhibitory Activity % ± S.D.**	
Chocolate without probiotics (Control)	**33.6%**	35.53 ± 1.03	34.79 ± 5.22	63.58 ± 0.38	61.33 ± 1.23
	54.5%	92.99 ± 1.37	84.47 ± 1.01	89.04± 1.49	87.46 ± 3.64
	70.5%	102.92 ± 14.19	99.72 ± 7.82	104.42 ± 5.58	99.84 ± 3.22
Chocolate with *A. muciniphila* DSM 22959	**33.6%**	38.11 ± 3.97	43.96 ± 11.03	58.84 ± 1.80	52.26 ± 11.03
	54.5%	88.88 ± 0.23	93.18 ± 2.52	88.88 ± 0.27	93.18 ± 4.05
	70.5%	105.23 ± 2.33	108.23 ± 10.48	105.23 ± 2.33	108.23 ± 10.48
Chocolate with *B. animalis* subsp. *lactis* BB-12®	**33.6%**	44.13 ± 0.88	43.90 ± 2.93	63.58 ± 0.71	67.11 ± 7.31
	54.5%	91.03 ± 2.79	90.50 ± 1.79	89.04 ± 3.02	91.01 ± 1.62
	70.5%	100.20 ± 2.85	96.89 ± 6.02	101.42 ± 1.44	103.09 ± 8.65

Annex 6

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Obrigada por participar neste teste triangular a chocolate.

Pedimos-lhe para realizar 2 ensaios com 3 amostras de chocolate codificado cada.

Por favor, avalie as três amostras ***do ensaio 1 pela ordem que se encontram no tabuleiro da esquerda para a direita*** *e assinale a amostra* ***diferente****.*
Caso não tenha notado diferenças assinale o código da amostra "diferente" com base no seu palpite.
Este teste é de resposta obrigatória.

Faça um pequeno intervalo e repita o procedimento para o ensaio 2

Nome do provador _____

Nº do tabuleiro _____

Ensaio 1 – amostra diferente _____

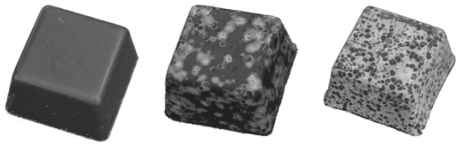
Ensaio 2 – amostra diferente _____

Obrigada pela sua participação.

Figure 2. 1 Triangular Test Form

Annex 7

Table 3. 13 Fat bloom and sugar bloom in chocolate

Condition	**Fat bloom**	**Sugar bloom**
Example		
Description	Development of fat bloom on chocolate. 1) Immediately after preparation; 2) After 12 days; 3) After 30 days	Bloom formation on poorly tempered chocolate
Reference	Kinta & Hatta, 2012	Kinta & Hartel, 2010