CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

AKKERMANSIA MUCINIPHILA AND DELIVERY SYSTEMS TO PROMOTE GUT HEALTH

by
Mariana Lopes da Fonseca

July 2023

CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

AKKERMANSIA MUCINIPHILA AND DELIVERY SYSTEMS TO PROMOTE GUT HEALTH

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
Mariana Lopes da Fonseca

Supervisor: Daniela Marlene da Silva Machado
Co-Supervisors: Ana Maria Pereira Gomes and Joana Cristina Pacheco Barbosa

July 2023

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

“Science never solves a problem without creating ten more.”

- George Bernard Shaw.

"Success is not final; failure is not fatal: it is the courage to continue that counts." -

Winston Churchill.

Resumo

Nos últimos anos, a espécie *Akkermansia muciniphila* emergiu como um probiótico de próxima geração, dadas suas relevantes atividades biológicas demonstradas em vários distúrbios intestinais e extraintestinais. Além disso, um dos principais desafios para a indústria tem sido desenvolver sistemas de entrega eficazes para garantir a sua elevada viabilidade e estabilidade durante a produção, tempo de prateleira do produto e após o consumo, especialmente ao longo do trato gastrointestinal. Com base nisso, esta tese teve como objetivo avaliar a potencialidade da *A. muciniphila* em promover a saúde intestinal, por meio de uma caracterização fenotípica de certas propriedades probióticas, e do desenvolvimento de sistemas de entrega que promovam a sua viabilidade durante o armazenamento sob refrigeração em aerobiose e quando exposto a condições simuladas do trato gastrointestinal.

Em primeiro lugar, foi realizada uma caracterização fenotípica da estirpe *A. muciniphila* DSM 22959, abordando as seguintes propriedades probióticas: hidrofobicidade, habilidades de agregação e melhoria da função de barreira intestinal. A estirpe mostrou baixa hidrofobicidade na superfície celular, enquanto as percentagens de auto-agregação e co-agregação foram semelhantes às obtidas para *Lacticaseibacillus rhamnosus* GG, estirpe controlo positivo. Em relação ao ensaio de integridade da barreira intestinal, não foi possível tirar conclusões devido à necessidade de otimização do protocolo utilizado. No entanto, há uma aparente resistência da *A. muciniphila* aos efeitos prejudiciais da *E. coli* sob as condições testadas. Após a caracterização fenotípica, a encapsulação por extrusão e a incorporação num alimento lácteo foram explorados como vetores tecnológicos para aumentar a viabilidade e estabilidade da *A. muciniphila* durante o armazenamento refrigerado em aerobiose e quando submetido ao trato gastrointestinal simulado. Verificou-se que, a espécie *A. muciniphila* foi encapsulada com sucesso na matriz de alginato de cálcio por extrusão (rendimento de 60%) e apresentou elevada estabilidade e viabilidade (aproximadamente 10^8 UFC/g) após 28 dias de armazenamento a 4ºC em aerobiose. Além disso, à medida que o tempo de armazenamento aumentou, a *A. muciniphila* encapsulada demonstrou maior viabilidade e estabilidade em condições gastrointestinais quando comparada à forma livre. A incorporação de *A. muciniphila* na matriz láctea composta por 77% (m/m) de requeijão português e 23% (m/m) de iogurte estilo grego originou um queijo creme probiótico com elevada qualidade microbiológica, baixo teor de fenólicos totais (cerca de 0,36 mg de equivalentes de ácido gálico/g de queijo seco) e importantes atividades biológicas, incluindo antidiabética (98.10% de inibição de α-glucosidase) e anti-hipertensiva (49.18% de inibição da Enzima Conversora de Angiotensina). Simultaneamente, o queijo creme garantiu um elevado nível de viabilidade de *A. muciniphila* (> 10^8 UFC/g) durante 21 dias de armazenamento a 4ºC em aerobiose e quando exposto a condições gastrointestinais. Além disso, apresentou um perfil semelhante em termos de textura, cor, atividade de água e pH, quando comparado com o queijo creme de controle/sem bactérias, sugerindo uma elevada aceitabilidade entre os consumidores.

Em conclusão, tanto a encapsulação na matriz de alginato de cálcio por extrusão como a incorporação em queijo creme parecem ser estratégias promissoras para proteger a viabilidade da *A. muciniphila* durante o armazenamento sob refrigeração em aerobiose e quando submetidas a condições gastrointestinais adversas.

Palavras-chave: *Akkermansia muciniphila*, queijo creme, extrusão, condições gastrointestinais simuladas, viabilidade.

Abstract

In the last years, *Akkermansia muciniphila* has emerged as a next-generation probiotic, given its demonstrated relevant biological activities in several intestinal and extra-intestinal disorders. Furthermore, one of the major challenges for industry has been to develop effective delivery systems to ensure its high probiotic viability during manufacturing, product shelf-life and after consumption, namely throughout the gastrointestinal passage. Based on the above, this thesis aimed to evaluate the potentiality of *A. muciniphila* to promote gut health, through a phenotypic characterization of certain probiotic properties, and to develop delivery systems that promote the viability of this probiotic throughout refrigerated aerobic storage and when exposed to simulated gastrointestinal conditions.

Firstly, a phenotypic characterization of *A. muciniphila* DSM 22959 strain was performed, concerning the following probiotic properties: hydrophobicity, aggregation abilities and improvement of intestinal barrier function. In this analysis, this strain showed low cell-surface hydrophobicity, whereas auto-aggregation and co-aggregation percentages were similar to those obtained for *Lacticaseibacillus rhamnosus* GG, positive control strain. Regarding the intestinal barrier integrity assay, no conclusions could be drawn due to the need for optimization of the protocol used, however there is an apparent bias of *A. muciniphila* against the harmful effect of *E. coli* under the conditions tested. Upon phenotypic characterization, encapsulation via extrusion and a dairy food vector were explored as technological strategies to enhance viability and stability of *A. muciniphila* during refrigerated aerobic storage and when submitted to gastrointestinal transit. Indeed, *A. muciniphila* was successfully encapsulated in a calcium-alginate matrix via extrusion (60% yield) and exhibited a high stability in viability (ca. 10^8 CFU/g) after 28-days of refrigerated aerobic storage. Moreover, as storage time increased, encapsulated *A. muciniphila* demonstrated higher viability and stability under gastrointestinal conditions when compared to its free counterpart. The incorporation of *A. muciniphila* in a dairy matrix based on 77% (m/m) "Requeijão" and 23% (m/m) Greek-style yogurt, originated a probiotic cheese spread with high microbiological quality, low total phenolic content (around 0.36 mg gallic acid equivalents/g of dried cheese) and interesting biological activities, namely antidiabetic (98.10% of α-glucosidase inhibition) and antihypertensive (49.18% of Angiotensin Converting Enzyme inhibition) properties. Simultaneously, this novel food ensured a high *A. muciniphila* viability level (> 10^8 CFU/g) during 21-days at 4ºC in aerobiosis and when exposed to gastrointestinal conditions. Additionally, this probiotic cheese displayed a similar profile in terms of texture, color, water activity and pH, when compared with cheese control/without bacteria, suggesting a potentially high acceptability among consumers. In conclusion, both extrusion in calcium-alginate matrix and incorporation into cheese spread seem to be promising strategies to safeguard *A. muciniphila* viability during refrigerated aerobic storage and detrimental gastrointestinal conditions.

Keywords: *Akkermansia muciniphila*, cheese spread, extrusion, simulated gastrointestinal conditions, viability.

Acknowledgements

First of all, I would like to thank professor Doctor Ana Gomes, my supervisor, for the opportunity and invitation to carry out this project at the Bioactive Microbiology Laboratory and for welcoming me into the Health and Nutrition research group. In addition, thank you for all the monitoring, guidance, availability, help and, above all, for believing in me.

To my supervisor, Doctor Daniela Machado, thank you for all the knowledge transmitted, advice given, late lunches and the countless hours spent helping me in the laboratory. Above all, thank you for your friendship and patience during this journey.

To my supervisor, Doctor Joana Barbosa, for her enormous ability to deal with problems, for her characteristic critical thinking, for all the advice offered, for all the availability, patience and above all for her friendship. Thank you very much.

To the master, Rita Vedor, thank you for being one of my greatest companions, for all the lunches and snacks spent around the same theme, and for the patience you had with me. Most of all, thank you for all the help and advice you have offered during this last year.

To my colleagues in the Bioactives Research Group, thank you for welcoming me with open arms and for making this journey better, thank you for all the help and advice offered and for all the knowledge transmitted.

To my friends, thank you for always being there for me, thank you for all the support, friendship and moments of joy you provided me.

To Tomás, thank you for your unconditional support, for all your patience and motivation throughout this journey. Thank you for always believing in me.

Finally, I want to thank my sister and my parents for being my pillars and for encouraging me to follow my dreams. Thank you for always believing in me and my potential.

Table of contents

Figure Index

Table Index

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

GIT – Gastrointestinal Tract

GRAS – Generally Recognized as Safe

IBD – Inflammatory Bowel Disease

IBS – Irritable Bowel Syndrome

LBP – Live Biotherapeutic Product

NGP – Next Generation Probiotic

QPS – Qualified Presumption of Safety

SCFA – Short Chain Fatty Acids

T2D – Type 2 Diabetes

Scientific outputs

Paper in Peer Reviewed Journals

Daniela Machado*, Mariana Fonseca*, Rita Vedor, Joana Cristina Barbosa and Ana Maria Gomes. Submitted. Calcium-alginate encapsulation of *Akkermansia muciniphila* via extrusion: viability and stability over aerobic storage and simulated gastrointestinal conditions. *Gels*. Submitted at 29th august.

Mariana Fonseca*, Rita Vedor, Daniela Machado, Joana C. Barbosa and Ana Maria Gomes. Submitted. Cheese spread incorporating *Akkermansia muciniphila*: physicochemical characterization, biological activities and probiotic viability during storage and simulated gastrointestinal passage. *Frontiers in Microbiomes*. Submitted at 4th september.

Poster presentation

D. Machado*, R. Vedor, M. Fonseca, M. Bento, J. C. Barbosa, D. Almeida, J. C. Andrade, A. M. Gomes (2022). Potential of *Akkermansia muciniphila* DSM 22959 and *Faecalibacterium duncaniae* DSM 17677 as live biotherapeutics for intestinal infections. Poster presented in 1st International Congress on Food, Nutrition & Public Health: Towards a sustainable future, Instituto Nacional de Saúde Doutor Ricardo Jorge, Lisboa, Portugal, 17th november 2022.

Fonseca, M.*; Machado, D.; Vedor, R.; Barbosa, J.C.; Andrade, J.C.; Gomes, A. M. (2023). Hydrophobicity and aggregation properties of gut commensals *Faecalibacterium duncaniae* DSM 17677 and *Akkermansia muciniphila* DSM 22959**.** Poster presented in Ciência 2023, Aveiro, 5th - 7th june 2023.

1. Introduction

1.1. Gut microbiota and health

Gut microbiota is defined as the assemblage of microorganisms present in the gastrointestinal tract and plays a vital role in the normal development and functioning of the human body and health (Ramirez et al., 2020). It is estimated that "the intestinal microbiota consists of a total number of 10^{13}-10^{14} microbial cells", considering autochthonous microorganisms and those that are ingested in food (Sánchez et al., 2017). The gut microbiota serves a crucial function in aiding the digestion and absorption of nutrients (Rowland et al., 2018). Some of these bacteria have the unique ability to break down complex carbohydrates and fiber that our own digestive enzymes cannot process. Consequently, they produce short chain fatty acids (SCFAs) as byproducts, which not only provide energy to the cells lining the colon but also contribute to overall gut health (Thursby & Juge, 2017). Moreover, the gut microbiota plays a critical role in training and regulating the immune system. It distinguishes between harmful pathogens and beneficial substances, preventing unnecessary immune responses and inflammation. Maintaining a diverse and balanced gut microbiota is essential for ensuring immune stability and effective defense against infections and diseases (Chen et al., 2021). In addition to supporting the immune system, a healthy gut microbiota acts as a protective barrier against harmful pathogens. It competes with potential invaders for resources and adhesion sites along the gut lining, making it challenging for pathogens to establish infections (Chen et al., 2021). Some gut bacteria even produce antimicrobial compounds that directly inhibit the growth of harmful microorganisms (Garcia-Gutierrez et al., 2019). An imbalance in gut bacteria composition, known as dysbiosis, has been associated with metabolic disorders like obesity and type 2 diabetes (DeGruttola et al., 2016). Specific gut bacteria can influence how our bodies extract and store energy from food, ultimately affecting weight management and overall metabolic health (DeGruttola et al., 2016). Moreover, certain gut bacteria are capable of synthesizing essential nutrients that may not be readily available in our diet. For instance, some gut microbes produce vitamins like B12, biotin, and K, which are vital for various physiological functions, including blood clotting, energy production, and maintaining healthy skin and hair (Rowland et al., 2018b; Wan et al., 2022). The gut-brain axis, a complex bidirectional communication system

between the gut and the brain, underscores the importance of gut microbiota in mental health. Gut microbiota significantly influences brain function, mood, and behavior by producing neurotransmitters and other signaling molecules that impact cognitive processes and emotional well-being (Chen et al., 2021). A disrupted gut microbiota has been associated with conditions like anxiety, depression, and stress-related disorders.

Lastly, a healthy gut microbiota plays a pivotal role in maintaining the integrity of the intestinal barrier, preventing harmful substances and toxins from leaking into the bloodstream (Di Tommaso et al., 2021). This barrier function is essential for safeguarding against inflammation and autoimmune reactions.

A complex interplay between alterations in bacterial diversity, colonization resistance capacity, epithelium integrity and other environmental factors may lead to dysbiosis of the gut microbiome. Such dysbiosis is closely related to a higher susceptibility to host bacterial infection. Commonly, the treatment for such infections entails the use of antibiotics (Dadonaite et al., 2018; Troeger et al., 2018). However, in the last decades, the overuse and misuse of antibiotics have led to an increase in antimicrobial resistance dissemination and the emergence of drug-resistant pathogenic bacteria, that may compromise the efficacy of antibiotic therapy (World Health Organization, 2021). Inappropriate use of antibiotics can lead to a reduction in the diversity of the gut microbiota, affecting its normal function. Thus, it can cause the development of bacterial resistance to antibiotics, altering their metabolic activity and increasing intestinal susceptibility to colonization (Lange et al., 2016). In addition, antibiotic administration increases the pool of resistance genes present in the gut microbiota (Rolain, 2013). Consequently, it becomes more difficult to treat infections due to the increased frequency of antibiotic-resistance bacteria. So, alternative strategies to fight bacterial infections are urgently required to overcome this situation, and probiotics have been pinpointed as a promising solution for diarrhea and related gut infections (Kopacz & Phadtare, 2022).

1.2. Probiotics: a promising solution for gut health

The modern history of probiotics starts in the 20th century, when Ellie Metchnikoff suggested that the bacteria inhabiting the intestinal tract provided beneficial effects on human health and promoted longevity in elder people (Metchnikoff, 1908). Later, in 1965, the scientists Lilly and Stilwell applied the concept of ‘probiotics’ to describe secreted substances produced by microorganisms that stimulate the growth of others

(Lilly & Stillwell, 1965). This concept suffered some changes in its definition over time and currently probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014).

For its classification as a probiotic, the microbial strains must fulfill certain criteria, namely in terms of i) safety parameters: isolation from suitable habitats, absence of pathogenic and virulence properties, as well as, lack of transmissible resistance genes conferring resistance to clinically used drugs; ii) functional properties: tolerance to the gastrointestinal conditions, properties of adhesion to the intestinal epithelium; antagonistic activity against pathogens; cholesterol reduction ability, anti-cancer effects and immunomodulatory properties and, in the best case scenario, ii) technological features: viability and beneficial properties must be maintained throughout processing, handling, storage and verified at the end of shelf-life within the product containing probiotic strains (World Health Organization & Food and Agriculture Organization of the United Nations, 2006). In Europe, probiotic safety is regulated by the European Food Safety Authority (EFSA), which has compiled a list containing the species which are safe for human consumption within a category known as – Qualified Presumption of Safety (QPS) (Binda et al., 2020). On the other hand, in the United States of America (USA), the generally recognized as safe (GRAS) system was established by the Food and Drug Administration (FDA) and is slightly different from the European approach (QPS), as the GRAS guidelines apply to food additives in general, while the QPS is focused only on microorganisms; GRAS addresses a specific substance or organism (working at the strain level), as opposed to the QPS system, which evaluates taxonomic units (often at the species level for bacteria and yeast, or at the family level for viruses). It should also be noted that GRAS status is determined by the FDA and/or external experts, while QPS status is determined only by EFSA (Barbosa et al., 2022). These probiotics can be provided to consumers as foods, dietary supplements, or drugs (Almeida et al., 2020; Barbosa et al., 2022).

As for the regulation of probiotic products, dietary supplements are intended to maintain or improve human health, while drugs are intended to have a therapeutic or preventive effect on the human body (Cordaillat-Simmons et al., 2020). In 2012, the FDA created a new category called live biotherapeutic product (LBP) to define "a biological product that: (1) contains live organisms, such as bacteria; (2) applies to the prevention, treatment, or cure of a disease or condition of human beings; and (3) is not a vaccine" (Food and Drug Administration, 2016). Like all products intended to prevent or treat disease, LBP

will have to be registered as medicines to reach the market in the USA and Europe. As such, they depend on the interaction between research institutions, pharmaceutical industries, and regulatory agencies (Barbosa et al., 2022; Cordaillat-Simmons et al., 2020).

The mechanisms of action involved in the beneficial effects exerted by probiotics are diverse, heterogeneous, varying from genus- to strain-specific, and in some cases remain not well elucidated (Plaza-Diaz et al., 2019). Nevertheless, four main mechanisms of action have been pointed out aligned with the beneficial properties of gut microbiota mention in section 1.1: 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, as illustrated in Figure 1 (Sánchez et al., 2017; Voss et al., 2022).

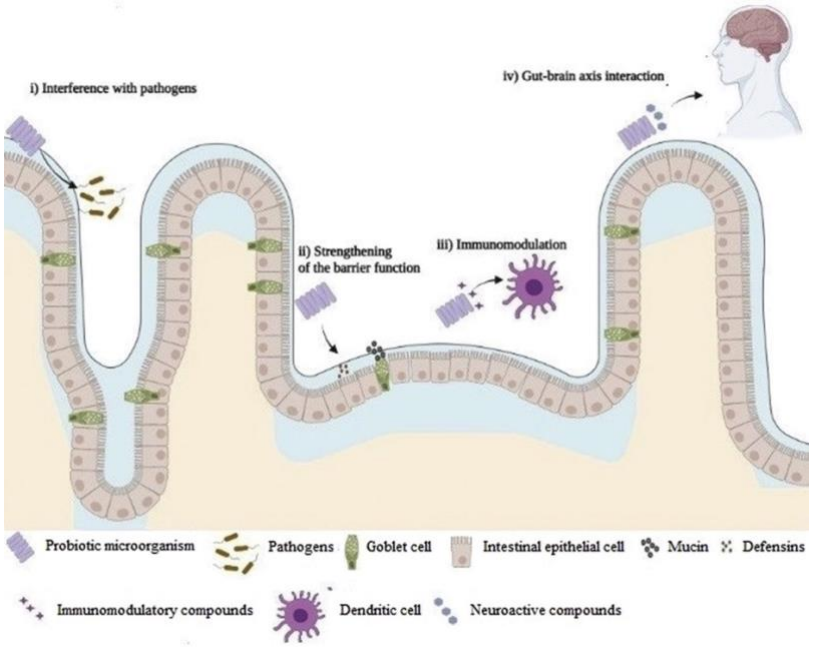

Figure 1.1 Mechanisms of action of probiotics. Representative scheme from Voss et al., 2022.

Currently, the well-characterized probiotic strains and, consequently, the most marketed belong to genera: *Bifidobacterium* and *Lactobacillus* [that suffered recent taxonomic changes being reclassified into 25 genera (Zheng et al., 2020)], and are commonly named as traditional probiotics (Almeida et al., 2020). Due to their extensive and safe use, classical probiotics have been listed as GRAS by FDA and QPS by EFSA (Martín & Langella, 2019). Nevertheless, certain conventional probiotics exhibit restricted impacts on the microbiota, indicating the need for an improved choice of microbial strains (Neef & Sanz, 2013). With the development of better culturing methodologies and increasing advances in genome and metagenome sequencing technologies, other species have been

discovered inclusively among intestinal commensal microorganisms, expanding the spectrum of potential probiotic strains (O'Toole et al., 2017). Among the more recent proposals, *Akkermansia muciniphila* was identified as a novel probiotic candidate, also termed as next-generation probiotic (NGP) (Andrade et al., 2020). It should be noted that these novel probiotics are known to have very restrictive survival conditions. Therefore, it is important to improve several features, including delivery vehicles, that can provide for high microbial viability (Almeida et al., 2020; Andrade et al., 2020). Additionally, studies regarding the efficacy, safety, physiological, genetic, and metabolomic characteristics of these microorganisms are needed for a full understanding of their potential (Andrade et al., 2020).

The following sections, 1.3 and 1.4, are focused on the intestinal commensal bacterial species *Akkermansia muciniphila* and they include a descriptive analysis of its physiological, microbiological characteristics, beneficial effects on health, and the available protective strategies to deliver this NGP.

1.3. *Akkermansia muciniphila* as a next-generation probiotic

In 2004, the species *Akkermansia muciniphila* was discovered during a search for a new mucin-degrading microbe in human feces at Wageningen University in the Netherlands (Derrien et al., 2004). *Akkermansia muciniphila* species belongs to the Verrucomicrobiota phylum and is a common resident bacterium of the gastrointestinal tract of humans and animals, being considered a promising probiotic candidate (Geerlings et al., 2018; T. Zhang et al., 2019). The high or low abundance of this bacterial species is positively related to host health and several disease states, including celiac disease (Verdu et al., 2015) , irritable bowel syndrome (IBS) (Distrutti et al., 2016), inflammatory bowel disease (IBD) (Sheehan et al., 2015) , and type 2 diabetes (T2D) (Schneeberger et al., 2015), respectively.

Microbiologically, *A. muciniphila* is characterized as an intestinal anaerobic, Gram-negative, oval-shaped, non-motile, and mucin-degrading bacterium (Derrien et al., 2004), corresponding to 1–3% of human fecal microbiota in healthy adults (Derrien et al., 2008). Originally, this microorganism was considered a strict anaerobe but recently it was demonstrated to exhibit a certain tolerance and resilience to aerobic environments, being reclassified as an aerotolerant anaerobe (Machado et al., 2020).

The distinctive feature of *A. muciniphila* lies in its capacity to enhance the regeneration and thickening of mucin, fortify the intestinal barrier, and decrease the permeability of microbial products within the intestine. This is achieved through the utilization of enzymes such as glycosyl hydrolases, proteases, sulfatases, and sialidase, which enable the breakdown of intestinal mucin glycoproteins. Subsequently, these components serve as the sole carbon source for SCFAs production, including acetate, propionate, 1,2-propanediol, succinate, and sulphate (Pellegrino et al., 2023). As depicted in Table 1.1, the administration of *A. muciniphila* has been related to several health benefits.

Table 1.1 In vivo studies demonstrating health benefits of *A. muciniphila* administration, published in 2022-2023.

Target conditions	Study type	Study Aim	Main findings	Reference
***Salmonella* Typhimurium infection**	Mice model	Investigation of the role of live and pasteurized *A. muciniphila* in protecting against the intestinal pathogen *Salmonella* Typhimurium in a mice model of infection treated with streptomycin.	The findings demonstrate that both forms decreased the amount of *Salmonella* present in the feces and systemic burdens, and reduced inflammation during infection.	(Liu et al., 2023)
Atopic dermatitis (AD)	Mice model	Evaluate the potential beneficial effect of *A. muciniphila* and *F. prausnitzii* bacteria in the treatment of atopic dermatitis.	Ingestion of each of these NGPs improved not only AD-related markers (dermatitis score, scratching behavior, and serum Ig E levels) but also balanced Th1 and Th2 immune responses.	(Y. Lee et al., 2022)
Muscular Atrophy	Mouse model	Evaluate the effect of ingestion of *A. muciniphila* and *F. prausnitzii* on muscle atrophy.	Probiotic supplementation improved grip strength but did not result in muscle mass gain.	(Byeon et al., 2022)

Periodontal and systemic inflammation	Mice model	Assess the effect of the administration of pasteurized *A. muciniphila* and Amuc_1100 on periodontal destruction in lean and obese mice.	It was demonstrated that 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)
Diabetes mellitus	Mice model	Analyze the protective effect of live and pasteurized *A. muciniphila* and the corresponding protein Amuc_1100 against diabetes mellitus.	Supplementation with *A. muciniphila* reduced body mass gain and plasma TNF-a levels. Additionally, this administration increased the number of goblet cells and mucin secretion.	(Deng Si-si et al., 2022)

***Clostridioides difficile* infection**	Mice model	Explore the protective effects of *A. muciniphila* on *Clostridioides difficile* inflammation (CDI) colitis.	*A. muciniphila* bacteria, through SCFA production, maintenance of bile acids, and modulation of the gut microbiome prevented weight loss and histological injury in the colon and alleviated symptoms of *Clostridioides difficile* inflammation.	(Wu et al., 2022)
Liver Injury	Mouse model	Evaluate the impact of consuming *A. muciniphila* combined with a high-fat diet on hepatic fibrosis.	Oral administration of *A. muciniphila* contributed to the restoration of the intestinal microbiota, due to strengthening of the intestinal epithelium and integrity, and to inhibition of liver inflammation.	(Raftar et al., 2022)

However, insufficient data is available on the potential of *A. muciniphila* to counteract pathogenic colonization. Only one study evaluated the potential inhibitory properties of *A. muciniphila* against a narrow spectrum of pathogenic bacteria. Indeed, Cozzolino et al. (2020) verified that *A. muciniphila* was able to auto-aggregate, form biofilm and co-aggregate with *Escherichia coli*, *Proteus mirabilis*, *Enterococcus faecalis,* and *Staphylococcus aureus*. Considering these findings, auto-aggregation and co-aggregation with pathogens seem to play a key role in the ability of *A. muciniphila* to antagonize pathogenic colonization. Notably, bacterial aggregation between microorganisms of the same strain (auto-aggregation) or between genetically different strains (co-aggregation) is of extreme relevance in several ecological niches, especially in the human intestine (Collado et al., 2008; Ferreira et al., 2011). To exert the desired benefit, probiotic strains should achieve an adequate mass through auto-aggregation (Krausova et al., 2019). Also, auto-aggregation has been related to the adhesion and biofilm formation abilities of probiotic strains, contributing to their survival and persistence in the intestine (Tuo et al., 2013). In turn, co-aggregation plays an important role in eliminating pathogens from the intestinal environment (Collado et al., 2008; Tuo et al., 2013). Thus, auto-aggregation properties and co-aggregation abilities with pathogens are routinely used for the preliminary selection of probiotic microbial strains (Collado et al., 2008; Krausova et al., 2019). Despite initial studies supporting the ability of auto- and co-aggregation of *A. muciniphila* with pathogens, we are far from understanding well the contribution of these aggregation properties, in preventing intestinal pathogenic colonization. Such scarcity of data urges further studies aiming to assess aggregation properties of *A. muciniphila* against a wide spectrum of intestinal pathogenic microorganisms, which may highlight the potential use of this NGP as a live microbial strategy to prevent and treat diarrhea and related intestinal infections.

1.4. Delivery systems involving *Akkermansia muciniphila*

Anaerobic commensal bacteria impose one of the major challenges in establishing production technologies and suitable delivery vehicles/formulations to ensure their viability in sufficient numbers, throughout production, distribution chain, and consumption until they reach the intestinal ecosystem, where they trigger health benefits. Thus, factors such as sensitivity to oxygen, low stomach pH levels, and release of bile salts impose challenges to the survival of anaerobic probiotics (Andrade et al., 2020). In

the case of *A. muciniphila*, Machado et al. demonstrated that when it was exposed to an aerobic environment at temperatures ranging from 4 to 37ºC it exhibited high oxygen tolerance up to 72 h and high stability, in terms of cultivable cell numbers when subjected to *in vitro* simulated gastrointestinal passage (Machado et al., 2020). Based on the demonstrated robustness, these findings suggest that *A. muciniphila* could be very attractive from a technological viewpoint for the development of pharmaceutical and food products. As shown in Table 1.2, some delivery systems involving *A. muciniphila* have been developed envisioning to promote its stability and viability under stress conditions such as aerobic storage and when exposed to gastrointestinal conditions.

Table 1.2 Studies reporting different delivery systems for *Akkermansia muciniphila*, main findings and associated limitations.

Delivery system	Main findings	Limitations/Drawbacks	Reference
Water-in-oil-in-water (W/O/W) double emulsion	*A. muciniphila* MUC^T (= DSM 22959, CIP 107961, ATCC BAA-835, JCM 33894) strain was efficiently encapsulated in a W/O/W double emulsion (97.5% of encapsulation efficiency). Furthermore, the encapsulated bacteria survival was higher than that of free dispersed cells after *in vitro* gastric and intestinal passage.	Encapsulated *A. muciniphila* suffered a dramatic reduction in viability after only 72 hours of storage at 4°C, either under aerobic or anaerobic conditions.	(van der Ark et al., 2017)
Encapsulation in spray-dried succinate-grafted alginate doped with epigallocatechin-3-gallate	*A. muciniphila* strain 139 was encapsulated via spray-drying in succinate-grafted alginate doped with an epigallocatechin-3-gallate matrix. This matrix protected *A. muciniphila* cells during spray-drying and the resulting formulation presented an improved storage in aqueous environments under anaerobic conditions at 4 °C for 12 days and showed an increased survival under simulated gastrointestinal conditions in comparison with spray-dried free cells.	A declining trend in viability was observed over the 12 days of anaerobic storage.	(Chang et al., 2020)

Spray-drying encapsulation using dairy-based matrices	*A. muciniphila* DSM 22959 encapsulated via spray-drying using 10% skim milk and inlet/outlet temperatures of 150/65 ºC ensure its viability higher than 7 log CFU/g during prolonged aerobic storage for 60 days and after exposure to simulated gastrointestinal passage.	Decreasing tendency in the viability of *A. muciniphila* spray-dried was observed over the 28 days of aerobic storage at both temperatures: 4ºC and 22ºC.	(Barbosa et al., 2022)
Encapsulation via emulsification/internal gelation using an alginate/denatured whey protein isolate matrix	*A. muciniphila* DSM 22959 was efficiently encapsulated in a sodium alginate/ denatured whey protein isolate matrix (64.4% entrapment efficacy). As storage time increased (0, 15, 30, 95 days), encapsulated *A. muciniphila* demonstrated higher stability in gastrointestinal conditions, when compared to its free counterpart.	The decrease in viable cell numbers was more evident for encapsulated bacteria than for free cells throughout prolonged storage for 95 days at both aerobic and anaerobic conditions.	(Almeida et al., 2022)

Encapsulation via extrusion in a xanthan and gellan gum matrix followed by freeze-drying	Marcial-Coba et al. (2018) encapsulated *A. muciniphila* DSM 22959 in a xanthan/gellan gum matrix, via the extrusion method, with a subsequent freeze-drying step, in which various combinations of cryoprotective agents were employed. These researchers verified that the cryoprotective solutions with a high sugar or protein content provided higher bacterial survival during freeze-drying. Furthermore, the survival rate of freeze-dried microencapsulated *A. muciniphila* was higher than that of free cells during *in vitro* simulated gastrointestinal passage.	A significant decrease in freeze-dried microencapsulated *A. muciniphila* viability was observed upon storage in both anaerobic and aerobic conditions, after 30 days, at both 4°C and 25 °C.	(Marcial-Coba et al., 2018)
Dark chocolate as a carrier for freeze-dried microencapsulated *A. muciniphila* in a xanthan/gellan gum matrix	Embedding in dark chocolate conferred increased protection to the encapsulated *A. muciniphila* since only a slight (although significant) reduction in viability was observed after 60 days of anaerobic storage at 4 °C and 15 °C. It also resulted in a high survival rate after *in vitro* gastric transit at pH 3.	Anaerobic storage application which does not correspond to a feasible storage modality (more expensive, not suitable for a household context).	(Marcial-Coba et al., 2019)

The lack of suitable carriers involving *A. muciniphila* reveals an urgent need for future studies aiming at the exploitation of novel delivery strategies for this NGP that provides simultaneously probiotic protection against gastrointestinal conditions and promotes viability and stability during aerobic storage. Effective protective strategies to enhance probiotic viability during storage and when exposed to harsh gastrointestinal conditions includes encapsulation techniques and incorporation in food matrices (Machado et al., 2020; Rolim et al., 2020). In particular, the extrusion encapsulation technique and incorporation of probiotics in dairy food matrices will be addressed in detail in the following sections, since these two approaches were applied in the present thesis.

1.5. Extrusion encapsulation of probiotics

Nowadays, the food and nutraceutical industries face a significant challenge in maintaining the viability of probiotic strains over extended periods when incorporating them into products that possess beneficial health properties upon consumption (Singh et al., 2022). The particular case of *A. muciniphila*, which has the potential to be used as a probiotic, requires the development of effective delivery systems. These systems are essential to safeguard the survival of this novel probiotic bacterium throughout the harsh conditions faced during manufacturing, distribution, shelf-life, and storage. Encapsulation has emerged as a promising technique with the ability to support the survival of probiotic bacteria within their unique environment, ensuring their arrival in the colon — the main target site — in sufficient quantities to deliver the intended health benefits (Andrade et al., 2020; J. Barbosa et al., 2022; Singh et al., 2022). Typically, encapsulation is defined as an entrapment process of substances (active ingredients) within another material (encapsulant) (Machado et al., 2020). One of the most commonly used encapsulating materials is alginate, since it exhibits interesting properties in terms of non-toxic nature, biocompatibility, biodegradability, easy handling, low in cost, capability to form strong gel structure through ionic crosslinking with calcium ions and pH responsiveness (i.e., it is stable at lower pH levels and unstable in higher pH conditions which is advantageous in tailoring release profiles) (Gheorghita Puscaselu et al., 2020; Koh et al., 2022). Among the various encapsulation techniques, extrusion is recognized as the oldest and most popular methodology to encapsulate probiotics, given its attractive characteristics namely, simplicity, employment of gentle conditions (no

involvement of extreme temperature, pH and organic/harmful solvents), low operational costs and high probiotic viability levels (Koh et al., 2022). In the extrusion process, probiotics are added and mixed into a hydrocolloid solution and, subsequently, the suspension is dripped into a hardening solution resorting to a syringe or nozzle (Koh et al., 2022; Xie et al., 2023). Indeed, previous studies demonstrated that the entrapment of probiotic bacteria in calcium-alginate capsules offered protection against harsh conditions encountered during storage or when subjected to *in vitro* gastrointestinal conditions (Chandramouli et al., 2004; Sousa et al., 2012).

1.6. Dairy food vector for probiotics delivery

Dairy products are commonly used as carriers for probiotics because they are widely accepted by consumers and have an excellent nutritional profile (Pinto et al., 2017; Rolim et al., 2020). Specifically, Greek-style yogurt consumption, also known as strained yogurt, is rapidly growing in the dairy industry due to its appealing texture and nutritional profile characterized by low-fat content and richness in proteins (Gyawali et al., 2022; Moineau-Jean et al., 2019; Yang & Yoon, 2022). By adding probiotic bacteria to strained yogurt, along with starter cultures, innovative probiotic yogurts with enhanced functional, physicochemical, sensory, and microbiological safety properties have been developed (de Morais et al., 2022; Yang & Yoon, 2022). Another excellent protective dairy matrix for probiotic cells is cheese. Its unique characteristics, including nutrient availability, fat and protein content that form a solid matrix structure with high buffering capacity, low oxygen levels, and high pH values, help safeguard probiotic microorganisms during storage and gastrointestinal passage (Kaur et al., 2022; Rolim et al., 2020).

In a circular economy perspective, whey cheeses have emerged as a sustainable approach to use whey, the main by-product of rennet and acid-coagulated cheesemaking (Bintsis & Papademas, 2023; Garcia et al., 2022). Whey cheeses are produced by denaturing whey proteins through heating at 88–92°C. These cheeses are produced worldwide and have different names depending on the country and region of origin (Bintsis & Papademas, 2023; Pintado et al., 2001). "Requeijão," has been identified as a valuable food matrix for incorporating probiotics due to its ability to support high survival rates of these beneficial microorganisms during storage and simulated gastrointestinal conditions (Garcia et al., 2022; Madureira, Amorim, et al., 2011; Madureira et al., 2006, 2008). Relevantly in this context, Faustino and colleagues developed a cheese spread incorporating red seaweed

Osmundea pinnatifida extract, using a dairy matrix comprising "requeijão" and Greek-style yogurt. This innovative dairy product was shown to have high microbiological quality and interesting bioactivities, mainly prebiotic and antihypertensive properties (Faustino et al., 2023).

Considering the whole state-of-the-art described in the previous sections, the objectives of this thesis were established and are described in the next topic.

1.7. Thesis aim

Akkermansia muciniphila has demonstrated great potential in the prevention and treatment of gut-related disorders. Nevertheless, there is a scarcity of information regarding the mechanisms involved in triggering of health benefits by this novel probiotic. Also, there are a lack of adequate technological solutions capable to offer a meaningful impact on the protection of *A. muciniphila* throughout aerobic storage and when exposed to detrimental gastrointestinal conditions. Based on the above rationale, this thesis (see Figure 1.2) aimed to i) characterize the strain *A. muciniphila* DSM 22959 in terms of certain probiotic properties namely cell-surface hydrophobicity, aggregation properties and promotion of intestinal barrier function, in comparison with *Lacticaseibacillus rhamnosus* GG, known as widely used and well-characterized probiotic strain and ii). design and study delivery systems for *A. muciniphila* able to enhance its viability and stability during aerobic storage and when exposed to gastrointestinal conditions. Bearing in mind the main goal of the thesis work, the study was divided into two parts with different specific objectives:

Part I - Phenotypic characterization of *A. muciniphila* DSM 22959 through the study of several physiological traits related to probiotic properties:

1) Evaluation of cell-surface hydrophobicity;

2) Assessment of auto-aggregation and co-aggregation properties with gut pathogens;

3) Assessment of the ability to promote intestinal barrier function.

Part II - Design and study of delivery systems based on an encapsulation technique and a food vector

4) Encapsulation of *A. muciniphila* DSM 22959 via extrusion method and evaluation of viability and stability of the encapsulated bacteria throughout 28-days under refrigerated aerobic storage and upon simulated gastrointestinal passage;

5) Development of a dairy food vector, incorporating *A. muciniphila* DSM 22959, based on “Requeijão” and Greek-style yogurt and their characterization in terms of:

5.1) Microbiological quality

5.2) Physicochemical properties including texture, color, pH, and water activity;

5.3) Total phenolic content and antioxidant, antidiabetic and antihypertensive activities;

5.4) Impact on viability and stability of *A. muciniphila* during refrigerated aerobic storage for 21-days and when exposed to *in vitro* simulated gastrointestinal conditions.

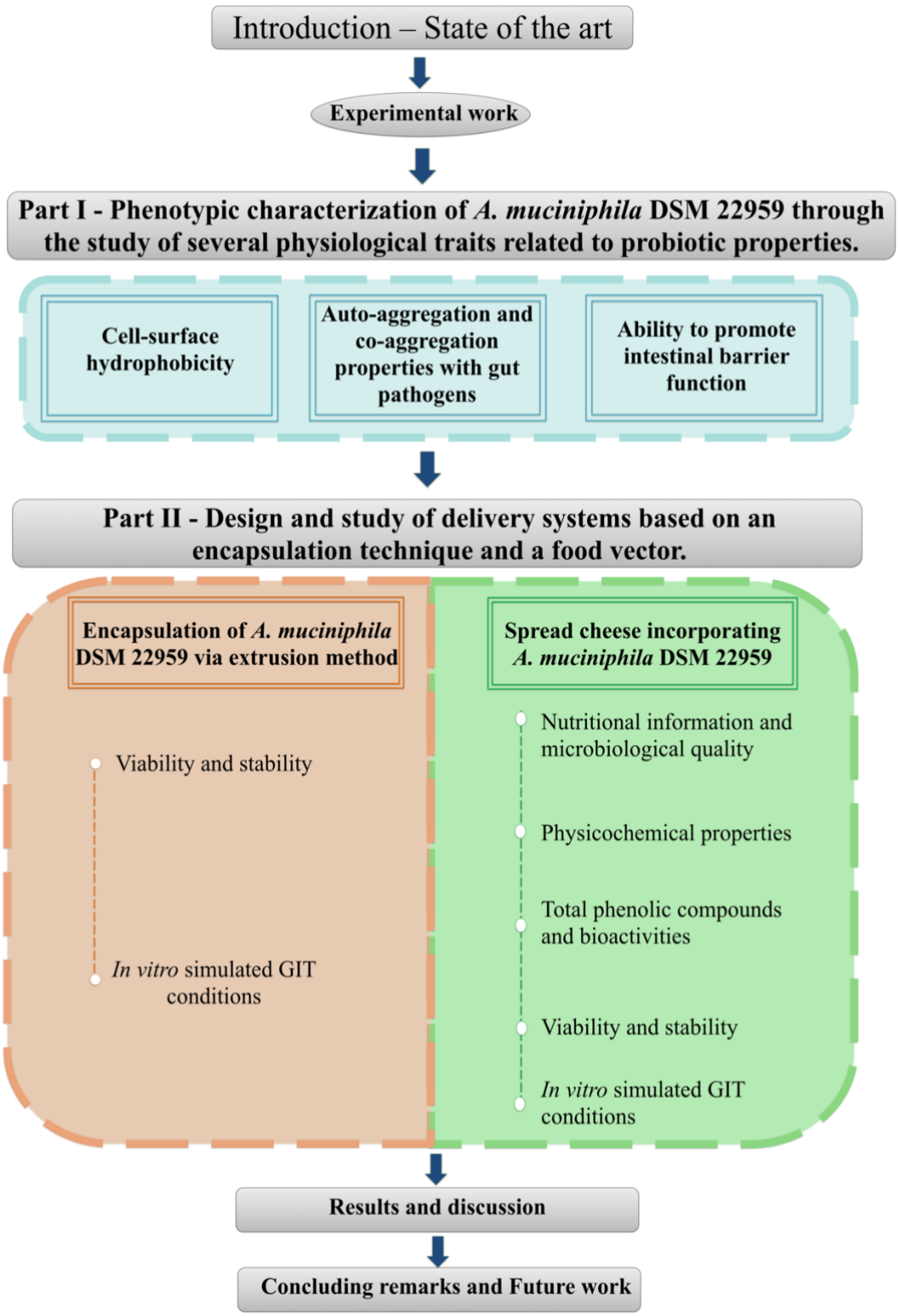

Figure 1.2 Schematic flow chart of thesis outline

2. Materials and methods

2.1. Bacterial strains culture conditions

Probiotic strains *Akkermansia muciniphila* DSM 22959 (*A. muciniphila* from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and *Lacticaseibacillus rhamnosus* GG (*L. rhamnosus* ATCC 53103, from ATCC - American Type Culture Collection, Manassas, VA, USA) were used in the present study. In turn, the bacterial pathogens used were: *Streptococcus intermedius* 2567 (from ESB - Escola Superior de Biotecnologia Culture Collection, Porto, Portugal), *Escherichia coli* O157:H7 (from ESB Culture Collection), *Escherichia coli* ATCC 25922 (from ATCC), *Klebsiella pneumoniae* ESB (from ESB Culture Collection), *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 (from ATCC), *Yersinia enterocolitica* NCTC 10460 (from NCTC - National Collection of Type Cultures, Salisbury, United Kingdom), *Staphylococcus aureus* DSM 11729 (from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures), *Staphylococcus aureus* ATCC 25923 (from ATCC) and *Listeria monocytogenes* NCTC 10357 (from NCTC).

All microbial strains were kept at -80ºC in proper media with 20% (v/v) glycerol (Fisher Scientific, Loughborough, UK) and sub-cultured at least twice before being used in the experiments. Specifically, *A. muciniphila* DSM 22959 was grown in PYG broth supplemented with 0.1% (m/v) mucin [PYGM, media composition as recommended by DSMZ (DSMZ, 2020) except that no resazurin was added] at 37ºC for 24 hours under anaerobic conditions (85% N_2, 5% H_2, and 10% CO_2) achieved in an anaerobic incubator (Whitley A35 HEPA anaerobic workstation, Bingley, UK). *Lacticaseibacillus rhamnosus* GG was grown aerobically in De Man, Rogosa, and Sharpe (MRS) broth (BIOKAR Diagnostics, Beauvais, France) at 37ºC for 20-24 hours. All pathogenic strains were grown aerobically in Trypto-casein soy broth (TSB, BIOKAR Diagnostics) at 37 ºC for 20-24 hours.

2.2. Hydrophobicity measurement

Cell surface hydrophobicity was measured according to the microbial adhesion to hydrocarbon method (MATH) described by Krausova et al., (2019) with some

modifications. Briefly, cells from *L. rhamnosus* GG were harvested by centrifugation (3850 *g*, at 4ºC, 10 minutes), and the same was performed for *A. muciniphila* but using different centrifugation conditions (12 000 *g,* at 4ºC, 30 minutes), to guarantee the total cell deposition and subsequent pellet formation. Afterwards, bacterial pellets were washed once with sterile phosphate-buffered saline (PBS, VWR Chemicals, Solon, OH, USA) and resuspended in PBS. The absorbances of bacterial suspensions at 600 nm (A600nm) were adjusted to 0.25 ± 0.05. Then an equal volume of n-hexane at 97% (Ibis Scientific, Las Vegas, NV, USA) was added and the mixture was vortexed for 1 minute. After phase stabilization and separation (10 minutes at room temperature), the A600 nm of the aqueous phase was measured (H) and the values of hydrophobicity (%) were calculated according to equation 2.1:

$$\text{hydrophobicity } \% = \left(\frac{H0-H}{H0}\right) \times 100 \qquad \text{Equation 2.1}$$

where H0 and H were the A600nm before and after extraction with n-hexane, respectively. According to their hydrophobicity percentage values, the bacterial strains were classified into high (71–100%), medium (36–70%), and low (0–35%), following the classification system proposed by Babot and colleagues (2014). These experiments were repeated three times on separate days and performed with technical duplicates per assay.

2.3. Auto- and co-aggregation evaluation

Auto- and co-aggregation abilities were evaluated via the spectrophotometric method according to Collado et al. (2008) and Jena et al. (2013) protocols, with minor modifications. Specifically for auto-aggregation assays, 10 mL of each bacterial suspension from *A. muciniphila* or *L. rhamnosus*, with A600nm adjusted to 0.25 ± 0.05 were prepared as described above for hydrophobicity experiments. These bacterial cell suspensions were incubated at 37°C in two atmospheric conditions: aerobiosis (ambient air) and anaerobiosis (atmosphere containing 85% N_2, 5% H_2, and 10% CO_2). In turn, for co-aggregation assays, equal volumes of probiotic (5mL) and pathogen (5mL) with A600nm adjusted to 0.25 ± 0.05 were mixed for at least 10 seconds and incubated at 37ºC in the same atmospheric conditions described for auto-aggregation assays. After 2 and 24 hours of incubation, the A600nm was measured, and the aggregation percentage was calculated according to the following equation 2.2:

$$\text{Aggregation } (\%) = \left(\frac{A0-At}{A0}\right) \times 100 \qquad \text{Equation 2.2}$$

where At represents the absorbance at different incubation times (2 and 24 hours) and A0 the absorbance in initial time (t=0). These experiments were repeated at least three times on separate days and included technical duplicates.

2.4. Intestinal barrier function assessment

2.4.1. Epithelial Cell Lines

The Caco-2 cells, derived from human colon adenocarcinoma, were obtained from ATCC. These cells were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM) at a temperature of 37°C in a humidified atmosphere with 5% CO_2. The DMEM was supplemented with 20% fetal bovine serum and a 1% antibiotic solution containing streptomycin/penicillin G and 1% of non-essential amino acids. The Caco-2 cells were grown to confluency in a transwell with an initial seeding density of 10^5 cells per well. The cells were allowed to grow for 15 days until a fully confluent cell monolayer was formed for experimental purposes.

2.4.2. Bacterial Strains and Culture Conditions

The probiotic strains *A. muciniphila* and *L. rhamnosus* and the pathogenic species *E. coli* O157:H7, used as the inflammatory agent, were grown under the same conditions as mentioned in section 2.2.1. All strains were adjusted to 10^8 CFU/mL to have the same number of cells as a starting point for all bacteria. For *in vitro* treatments, the desired bacterial count (10^8 CFU/mL) was suspended in DMEM without any antibiotics or supplements.

2.4.3. Assessment of *Akkermansia muciniphila* influence on the permeability of an epithelial barrier model

For the determination of the phenol red dye flow and TEER assays the protocol developed by Bhat et al. (2020) was followed with minor modifications. These assays were used to evaluate the effect of intestinal barrier permeability of *L. rhamnosus* (control), *A. muciniphila* and *E. coli* using Caco-2 cells. Transepithelial Electrical Resistance (TEER) and phenol red diffusion assay were determined before and after pathogen exposure. Note that the probiotic species *L. rhamnosus* GG was used for comparative purposes.

2.4.3.1. Phenol Red Flux Assay

The determination of phenol red flux was assessed based on the protocol described by Bhat et al. (2020), with slight modifications. Upon the establishment of differentiated Caco-2 cell monolayers in transwell culture inserts, the phenol red diffusion assay was conducted as follows. The apical and lower chambers were initially washed with PBS. Subsequently, 200 µL of phenol red-containing DMEM was added to the upper chamber, while 900 µL of phenol red-free DMEM was added to the basal compartment. The two-chamber culture system was then incubated at 37°C in a humidified incubator with CO_2 for 1 hour. To assess monolayer integrity, the diffusion of phenol red was determined by measuring the absorbance of the basolateral medium at 558 nm after washing with PBS. The extent of diffusion was expressed as the percentage of phenol red that diffused into the basal chamber from the apical chamber.

To investigate the impact of *A. muciniphila* on intestinal cell monolayer integrity in the presence of the inflammatory agent *E. coli*, Caco-2 cells were subjected to various assays: exclusion (Ex), competition (Com), and displacement (Dis), as follows: In the exclusion assay, Caco-2 cells were pre-treated with *A. muciniphila* for 3 hours, followed by exposure to *E. coli* for another 3 hours. The same procedure was performed for *L. rhamnosus* GG. In the competition assays, both the probiotic and *E. coli* were incubated simultaneously with Caco-2 cells for 3 hours. Conversely, in the displacement assay, Caco-2 cells were initially exposed to *E. coli* for 3 hours, after which they were incubated with the probiotic *A. muciniphila* for the same duration. The same procedure was performed for *L. rhamnosus* GG. Additionally, Caco-2 cells were treated individually with *A. muciniphila*, *L. rhamnosus* GG, and *E. coli*, as control. Caco-2 cells without any treatment were used as integrity control. The results are expressed in permeability percentage and were calculated according to equation 2.3:

$$\% \text{ Permeability (Phenol red flux)} = \frac{Abs_{sample}}{Abs_{control}} \times 100 \qquad \text{Equation 2.3}$$

where Abs_{sample} corresponding at absorbance of the sample and $Abs_{control}$ corresponding at absorbance of the control with phenol red.

2.4.3.2. Transepithelial Electrical Resistance (TEER) Assay

To evaluate gut barrier integrity, the measurement of transepithelial electrical resistance (TEER) was performed across the monolayer of intact Caco-2 cells using the Millicell® ERS-2 Voltohmmeter (electrical resistance system, Millicell, Merck, Germany). Fully differentiated Caco-2 cells with TEER values above 300 $\Omega*cm^2$ were selected for the experiment. Individual treatments with *L. rhamnosus*, *A. muciniphila*, and *E. coli* were performed on the Caco-2 monolayers, as described previously for the phenol red permeability assay. In each set of experiments, a control group of Caco-2 cells without any treatment was included. TEER measurements were obtained before and after the treatment. The TEER values obtained after treatment were divided by the initial TEER values when no probiotic or inflammatory agent was added. This ratio, referred to as the sample TEER (TEERS) on treatment, was used to assess the impact of each treatment on gut barrier integrity.

2.5. Encapsulation of *Akkermansia muciniphila* DSM 22959 via extrusion in calcium-alginate matrix

2.5.1. Inoculum preparation

Akkermansia muciniphila was grown as aforementioned (section 2.1.1), and the cells were harvested by centrifugation at 12 000 × g for 30 min at 4°C (Sorvall LYNX 4000 Superspeed Centrifuge, Thermo Scientific, MA, USA) and washed once with physiological saline solution [0.85% (m/v) NaCl]. After centrifugation, the pelleted biomass was resuspended in physiological saline solution and adjusted to a cell concentration around 10^9 CFU/mL. This resulting bacterial suspension was either used directly as free cell control or for encapsulation procedure via extrusion (5 mL).

2.5.2. Extrusion procedure

Encapsulation via the extrusion technique was based on Sousa et al. (2012) with some modifications. Briefly, *A. muciniphila* saline suspension (with a concentration of around 10^9 CFU/ml) was added at 10% (v/v) to 2% (m/v) sodium alginate (Sigma-Aldrich, St. Louis, MO, USA). Afterwards, the alginate-culture mixtures (in a proportion of 45mL alginate:5 mL bacterial suspension) were loaded in a 60 mL syringe (Enfa, Jiangsu

Kanghua Medical Equipment Co. Ltd., Jiangsu, China) coupled with a syringe needle (21 G × 1.5″, ICOplus 3, KD Medical GmbH Hospital Products - Berlin, Germany). This mixture (50 mL) was then extruded into 200 mL of 4% (m/v) $CaCl_2$ (Sigma-Aldrich, USA) solution stirred at 200 rpm (Heidolph MR 3001 magnetic stirrer; Heidolph Instruments GmbH & Co. KG, Schwabach, Germany). The extrusion rate was 4.0 mL/min. The flow rate was controlled using a syringe pump (Braintree Scientific BS-300 syringe pump, Braintree Scientific, MA, USA). Afterward, the resulting capsules were left in contact with the $CaCl_2$ solution for 30 minutes at room temperature to ensure complete solidification. The $CaCl_2$ solution was subsequently removed by decanting and the capsules were suspended in physiological saline solution. Lastly the capsules were recovered using a fine mesh strainer and they were stored according to conditions described below in section 2.5.5.

2.5.3. Enumeration of free and encapsulated *Akkermansia muciniphila* cells

For the enumeration of free *A. muciniphila* cells, decimal dilutions were performed in PBS and spotted, in triplicate, on PYGM agar plates (PYGM broth supplemented with 1.5 % (m/V) agar (BIOKAR Diagnostics). Plates were incubated for 5-7 days at 37°C under anaerobic conditions, and results were expressed in colony-forming units per milliliter (CFU/mL). Concerning the encapsulated bacteria, the capsules were suspended in a tri-sodium citrate dihydrate (Merck KGaA, Darmstadt, Germany) solution at 2.28% (w/v) in a 1:9 (g/mL) ratio and subjected to the mechanical action of sterile pellet pestle (Sigma-Aldrich, St Louis, MO, USA) assisted with vortexing for 5 minutes to allow the complete release of *A. muciniphila* cells from capsules. This treatment was previously tested, and it was shown not to affect the viability of *A. muciniphila* cells (unpublished results). The resulting suspension was then serially diluted, as described for the free cells, and the results were expressed as CFU/g.

2.5.4. Encapsulation yield calculation

Encapsulation yield (EY) is a combined measurement of the efficacy of entrapment and survival of viable cells during the encapsulation procedure, and it was calculated according to the formula proposed by Martin et al. (2013) (equation 2.4):

$$\text{EY}\ (\%) = \left(\frac{\text{N}}{\text{N0}}\right) \times 100 \qquad \text{Equation 2.4}$$

where N is the number of CFU released from capsules and N0 is the number of CFU present in bacterial suspension added to the alginate solution during the encapsulation procedure.

2.5.5. Viability of free and encapsulated *Akkermansia muciniphila* cells during refrigerated aerobic storage

The effect of encapsulation via extrusion on *A. muciniphila* viability was assayed, in comparison to free cells, throughout refrigerated aerobic storage for 28-days. Free cells were stored as follows: equal portions (1 mL) of cell suspension were stored at 4ºC under aerobic conditions into 10 sterile microtubes, corresponding to two replicas for each sampling timepoint. Concerning the encapsulated bacteria, capsules were weighed into 50 mL sterile centrifuge tubes and suspended in physiological saline solution in a 1:9 (g/mL) ratio; these preparations were performed in duplicate for each sampling timepoint, and subsequently stored at 4ºC under aerobic conditions. The viability of free and encapsulated *A. muciniphila* cells was evaluated on the day of encapsulation and after 7, 14, 21 and 28-days of refrigerated aerobic storage, following the protocol previously described in section 2.5.3.

2.5.6. Resistance of free and encapsulated *Akkermansia muciniphila* cells to *in vitro* simulated gastrointestinal passage

The viability of free and encapsulated *A. muciniphila* cells when exposed to *in vitro* simulated gastrointestinal conditions was determined at 1 and 28-days after the extrusion procedure using a standardized digestion method described by Brodkorb et al. (2019) with minor changes. Briefly, either 0.5 mL of free cells in 0.85% (m/v) NaCl or 0.5 g of calcium-alginate capsules were distributed into independent tubes (two replicates per timepoint and condition). To simulate the temperature and peristaltic movements of the

human digestion, an orbital shaker incubator (Wiggen Hauser, Berlin, Germany) was used at 37°C and 200 rpm. For each assay, all enzyme solutions were freshly prepared. For the gastric phase, samples were exposed to 2 mL of simulated gastric fluid (pH 3), containing pepsin (2000 U/mL—from porcine gastric mucosa; Sigma Aldrich, St. Louis, MO, USA) for 2 h. Afterwards, intestinal conditions were simulated for 3 h at pH 7, by adding 4 mL of simulated intestinal fluid containing pancreatin (based on the trypsin activity at 100 U/mL in the final mixture; Sigma Aldrich, St. Louis, MO, USA) and bile salts (Sigma Aldrich, St. Louis, MO, USA). To evaluate the effect of gastric and intestinal conditions on *A. muciniphila* viability (free and encapsulated), samples were collected at the end of each phase (gastric and intestinal) and cell enumeration was performed according to the procedure previously described in section 2.5.3. Note that *in vitro* digestion protocol was performed under an aerobic atmosphere, while the PYGM agar plates were incubated under anaerobic conditions.

2.6. Cheese spread incorporating *Akkermansia muciniphila* DSM 22959

2.6.1. Cheese spread production

Commercial whey cheese from cow's milk "Requeijão" and Greek-style yogurt (without added sugar) were purchased from a local supermarket (Lidl, Porto, Portugal). The nutritional declaration of both is presented in Table 2.1. The recipe for the cheese spread was based on the previous work of Faustino et al. (2023) with some modifications: 77% (m/m) of pasteurized whey cheese and 23% (m/m) of the Greek-style yogurt. Briefly, 27 independent cheese spreads were prepared in sterile container by mixing 21 g of whey cheese with 6 g of Greek-style yogurt per cheese spread. Afterwards, all 27 cheese spreads were subjected to thermal treatment (10 min at 90ºC) in a water bath (GFL Gesellschaft für Labortechnik mbH, Burgwedel, Germany). The pasteurized cheese spreads were left to cool down to room temperature. Then, the *A. muciniphila* pellet (ca. 3 x 10^{10} CFU) was incorporated into 15 of the 27 cheese spreads by mixing well with a sterile spatula, allowing to obtain fifteen cheese spreads with *A. muciniphila*. Note that the *A. muciniphila* pellet was obtained by centrifugation of the 30 mL of *A. muciniphila* saline suspension (with a concentration of about 10^9 CFU/mL) at 12000 g for 30 min with subsequent elimination of the supernatant. The remaining twelve processed cheese

spreads without *A. muciniphila* were included in the experiments as control cheese samples.

Table 2.1 Nutritional declaration of "Requeijão" and Greek-style yogurt per 100g.

Nutrition declaration per 100g	Requeijão	Greek-style yogurt
Energy	585 kJ/140kcal	442 kJ/107 kcal
Fat	9.4 g	8.5 g
of which saturates	6.7 g	5.4 g
Carbohydrates	5.1 g	4.3 g
of which sugars	4.0 g	3.6 g
Proteins	8.9 g	3.2 g
Salt	0.63 g	0.11 g

2.6.2. Microbiological evaluation of cheese spread

To assess the viability and stability of *A. muciniphila* in the cheese spread, as well as to identify potential microbial contaminations, microbiological analyses were conducted on three randomly selected cheese spreads. These analyses were performed on the day of production (day 0) and after 7, 14, and 21 days of refrigerated (at 4ºC) aerobic storage. For the analysis, a sample weighing 1 gram from each cheese spread was diluted in 9 mL of PBS and homogenized using a vortex. Serial decimal dilutions were then prepared in PBS and plated in triplicate on various solid media. PYGM agar for enumeration of *A. muciniphila,* de Man, Rogosa and Sharpe agar (MRS, BIOKAR Diagnostics, Beauvais, France) for the counting of lactic acid bacteria, plate count agar (PCA, Merck, Darmstadt, Germany) for the enumeration of mesophilic aerobic bacteria, potato dextrose agar (PDA, BIOKAR Diagnostics, Beauvais, France) for the counting of yeasts and molds, and violet red bile glucose agar (VRBGA, BIOKAR Diagnostics, Beauvais, France) for the detection and enumeration of Enterobacteriaceae. The inoculated agar plates were then incubated under specific conditions: PYGM agar at 37ºC anaerobically for 3-7 days, MRS agar at 37°C under both aerobic and anaerobic conditions for 2 days, PCA at 30°C aerobically for 2-5 days, PDA at 20ºC aerobically for 5 days, and VRBGA at 37°C aerobically for 2 days. After the respective incubation periods, colony numbers were counted, and the results were expressed as CFU per gram (CFU/g) of cheese spread.

2.6.3. Physicochemical characterization of the cheese spread

One day after production, the physicochemical parameters including texture, color, water activity, and pH were analyzed, in triplicate, for both the cheese spread incorporating *A. muciniphila* and the control cheese spread (i.e., without *A. muciniphila*).

Texture properties were evaluated by measuring the force-time curve using a TA.XT apparatus (Stable Micro Systems, Surrey, UK). An extrusion test was performed, as suggested by the manufacturer for this type of dairy product. Briefly, a back extrusion rig (A/BE) composed of a sample container and a disc plunger with 40 mm were used. The container was filled up to 75% of its capacity, to exclude base interferences. The disc plunger performs a compression test which extrudes the product up and around the edge of the disc. This test measures the consistency of viscous products, such as yoghurt, creams and sauces, and the results relate to measurements of viscosity. The disc plunger was attached to a 5 kg load cell, which was calibrated using a 2 kg weight. The extrusion was performed in triplicate at room temperature (22 ± 2ºC). Distance was set at 30 mm with a trigger force of 0.5 g and a test speed of 1 mm/s. This test allowed the measurement of various attributes such as firmness, consistency, and cohesiveness.

The color evaluation was performed using a colorimeter (Chroma meter CR 400, Konica Minolta, Osaka, Japan). The device provided values for lightness (L*), green-red chromaticity coordinate (a*), and blue-yellow chromaticity coordinate (b*). The color differences (ΔE*) between the cheese spread with *A. muciniphila* and cheese spread control were calculated using the equation 2.5:

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{(1/2)} \qquad \text{Equation 2.5}$$

where ΔL*, Δa*, and Δb* are the difference between the two samples (cheese spread with *A. muciniphila* and cheese spread control) in L*, a*, and b*, respectively. The perception of the color difference ΔE* varies according to the observed color and the sensitivity of the human eye. The minimum detectable color difference for the human eye is when ΔE* is at least between 1–3 (Bodart et al., 2008).

The water activity (a_w) of the cheese spreads was analyzed using a water activity meter (LabMaster-aw neo, Lachen, Switzerland). The pH measurements were taken by immersing the electrode probe of a pH meter (Basic 20 Crison Instruments, Barcelona, Spain) into the cheese spreads.

2.6.4. Extract preparation for total phenolic content, antioxidant, antidiabetic, and antihypertensive activities determination

Lyophilized cheese spread extracts were prepared from both cheese spread with *A. muciniphila* and control cheese spread according to the procedure described by Ribeiro et al. (2021) with minor modifications. Briefly, cheese spread with *A. muciniphila* and control cheese spread samples (each in triplicate) were homogenized with 30 mL of methanol acidified with formic acid (9:1 v/v), using an orbital shaker (Wiggen Hauser, Berlin, Germany) at 250 rpm, for 1 h. The homogenized samples were centrifuged at 3850 ×g, at 4 ºC for 10 min (Hettich Universal 320R Centrifuge, Andreas Hettich GmbH & Co. KH, Tuttlingen, Germany), and the supernatant was kept at -20 ºC overnight, to allow for protein precipitation. The resulting slurry was centrifuged again using the previous conditions to remove remaining soluble proteins. The extract was evaporated using a rotavapor (Buchi, Flawil, AL Switzerland) under the following conditions: bath temperature of 45ºC, pressure of 100 atm, for approximately 30 minutes. Lastly, the final volume of each extract was adjusted to 5 mL, by adding deionized water.

2.6.5. Determination of the total phenolic content

The total phenolic content for cheese spread with *A. muciniphila* and cheese spread control was determined using the Folin-Ciocalteu colorimetric method, following the protocol described by Singleton & Rossi (1965) and Coscueta et al., (2018) with minor modifications. To express the results in milligrams of gallic acid equivalents per milliliter of the sample (mg GAE/mL), a calibration curve for gallic acid, ranging from 0.025 to 0.200 mg/mL, was prepared.

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 designated well. The microplate was wrapped in aluminum paper and incubated in the dark at 25°C for 30 minutes. The resulting blue mixtures were read at 765 nm using a multi-detection plate reader (Synergy H1, VT, USA) operated with the Gen5 software. The results were expressed as milligrams of gallic acid equivalent per gram of dried cheese spread (mg GAE/g). All assays were performed in triplicate.

2.6.6. 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) scavenging assay

The antioxidant activity was evaluated using the ABTS (2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) scavenging assay described by Gonçalves et al., (2009) with slight modifications. Initially, the concentration of the ABTS working solution was adjusted to achieve an initial absorbance of 0.70 (± 0.02) at 734 nm. Afterward, a Trolox solution was prepared by dissolving 0.0125 g of Trolox (Sigma-Aldrich, MO, USA) in 1 mL of methanol (Fischer Chemical, MA, USA), and the volume was completed to 50 mL with ultra-pure water. Then, a calibration curve of Trolox (25-175 µM) was prepared to allow the expression of the results as µmol of Trolox equivalents per gram of dried sample. For the assay, 20 µL of Trolox, sample, or solvent and 180 µL of the ABTS working solution were added to each well of a 96-well microplate. The microplate was incubated for 5 minutes at 30°C and the absorbance at 734 nm was measured using a multi-detection plate reader (Synergy H1, VT, USA). All assays were performed in triplicate.

2.6.7. α-Glucosidase inhibition assay

The antidiabetic activity was evaluated using the α-glucosidase inhibitory activity assay described by Kwon et al., (2008) with minor changes. Briefly, 50 µL of the samples were mixed with 100 µL of 0.1 M phosphate buffer (pH=6.9) containing α-glucosidase solution (1.0 U/mL) in each well of a 96-well microplate. The mixture was incubated at 25°C for 10 minutes. Subsequently, 50 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH=6.9) was added to each well. 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, VT, USA) at 405 nm.

For this assay, a negative control containing 50 µL of buffer solution in place of the sample and a positive control containing 50 µL of acarbose at a concentration of 10 mg/mL were used. All assays were performed in triplicate cheese spread samples. The α-glucosidase inhibition was calculated using the following equation 2.6:

$$\alpha - Glucosidase\ inhibition\ (\%) = \left(\frac{\Delta Abs_{control} - \Delta Abs_{sample}}{\Delta Abs_{control}}\right) x\ 100 \quad \text{Equation 2.6}$$

where $\Delta Abs_{control}$ is the variation of absorbance of the control and ΔAbs_{sample} is the variation of absorbance of the cheese spread samples.

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

The antihypertensive activity was determined using the ACE-inhibitory activity assay, following the protocol described by Sentandreu & Toldra (2006), with slight modifications. Initially, 40 µL of ultrapure water or ACE working solution (42 mU/mL) were added to the respective wells. The final volume of 80 µL was adjusted by adding ultrapure water to the blanks and the respective samples to the color control, or sample wells. Subsequently, the enzymatic reaction was initiated by adding 160 µL of substrate solution (0.45 mM), and the mixture was incubated at 37°C. After 30 minutes, the generated fluorescence was measured using a multi-detection plate reader (Synergy H1, VT, USA) with excitation and emission wavelengths set at 350 nm and 420 nm, respectively. All assays were performed in triplicate.

For the calculation of ACE inhibitory activity (iACE), the following equation 2.7 was used:

$$iACE\ (\%) = \left((F_{CTL} - F_{BLK}) - (F_{SPL} - F_{SPLB})\right) * \frac{100}{F_{CTL} - F_{BLK}} \qquad \text{Equation 2.7}$$

where F_{CTL} represents the fluorescence intensity of the control, F_{BLK} represents the fluorescence intensity of the blank, F_{SPL} represents the fluorescence intensity of the sample, and F_{SPLB} represents the fluorescence intensity of the color sample control.

2.6.9. *In vitro* simulated gastrointestinal passage of cheese spread incorporating *A. muciniphila*

The survival capacity of *A. muciniphila* incorporated in cheese spread and the respective free cells counterpart when exposed to *in vitro* simulated gastrointestinal conditions was determined for cheese spreads upon 1 and 21-days of manufacture and subsequent storage under refrigeration at 4ºC, using the same *in vitro* digestion protocol described for encapsulated *A. muciniphila* cells in section 2.5.6.

2.7. Statistical analysis

Data were expressed as the mean ± standard deviation (SD) of replicates and analyzed using IBM SPSS Statistics 28.0 software (Chicago, IL, USA). Parametric tests were performed if the data followed a normal distribution according to the Shapiro-Wilk test (normality test).

Thus, for the statistical analysis of probiotic properties in terms of hydrophobicity, auto-aggregation, co-aggregation with pathogens and promotion of intestinal barrier function by *A. muciniphila* when compared with *L. rhamnosus* GG, as well as in the comparison of cheese spread with *A. muciniphila* with control cheese spread in terms of texture, water activity, pH and bioactivities, the t-student test for independent samples was used. Statistical differences were considered significant with P values < 0.05.

3. Results and Discussion

Akkermansia muciniphila, a highly prevalent bacterial species within the intestinal microbiota, has garnered great attention from the scientific community due to its potential role in enhancing gut health. Numerous studies have highlighted the advantages of administering *A. muciniphila*, as mentioned in Table 1.1. However, there is a lack of strategies that can safeguard *A. muciniphila* against adverse conditions encountered during aerobic storage and when subjected to gastrointestinal transit. Hence, an in-depth physiological characterization of *A. muciniphila* DSM 22959 regarding certain probiotic properties, including, hydrophobicity, auto-aggregation, co-aggregation with pathogens, and ability to promote gut barrier integrity, was performed and will be discussed in sections 3.1-3.3. Furthermore, the two delivery systems that were developed for *A. muciniphila*, aiming to enhance its viability and stability during aerobic storage and exposure to gastrointestinal conditions, will be discussed in sections 3.4-3.5.

3.1. Cell surface hydrophobicity

Microbial affinity to nonpolar solvents, such as xylene, toluene, or n-hexane, has been frequently used to determine the hydrophobicity of probiotic candidates. This parameter helps to predict their potential adhesion to the host's cells and subsequent successful colonization by these beneficial microorganisms (Collado et al., 2008; Cozzolino et al., 2020; Krausova et al., 2019). As it can be observed in Figure 3.1, *L. rhamnosus* GG exhibited a statistically significant ($p<0.05$) higher hydrophobicity percentage (61.65 ± 9.20%) than *A. muciniphila* DSM 22959 (28.95 ± 2.83%). Furthermore, according to the hydrophobicity classification system proposed by Babot et al. (2014), *L. rhamnosus* GG and *A. muciniphila* DSM 22959 may be classified as bacterial strains displaying medium (hydrophobicity % > 36%) and low (hydrophobicity % < 35%) hydrophobicity, respectively (Fig. 3.1). It should be noted that previous studies reported hydrophobicity values for *A. muciniphila* DSM 22959 and *L. rhamnosus* GG ranging ca. 6-44% and 44-64%, respectively. These oscillations can be attributable to the solvent employed (xylene, toluene, or n-hexane) and the solvent contact time (15-60 min) (Collado et al., 2008; Cozzolino et al., 2020; Tuo et al., 2013). Despite the variations in the procedures used for hydrophobicity determination between studies, it is possible to observe a trend, that is, *A. muciniphila* tends to present lower hydrophobicity than *L. rhamnosus* GG.

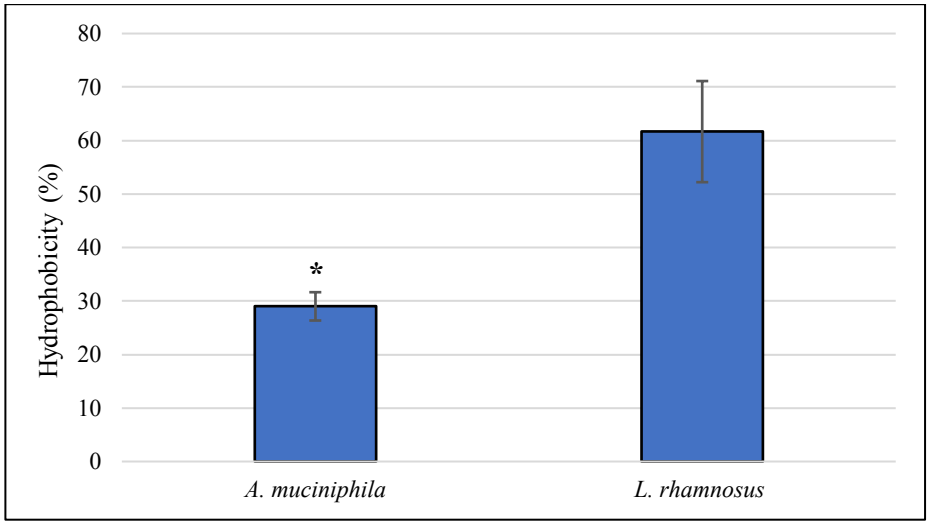

Figure 3.1 Hydrophobicity percentages of *L. rhamnosus* GG and *A. muciniphila* DSM 22959. Error bars represent standard deviation of the mean of 3 independent experiments. The symbol * indicates the statistically significant differences ($p < 0.05$) between *A. muciniphila* DSM 22959 strain in comparison to the data obtained for *L. rhamnosus* GG.

3.2. Auto- and co-aggregation properties

The ability of bacteria to adhere to surfaces is crucial for their establishment in the intestines (Cozzolino et al., 2020). Auto-aggregation is defined as the ability of microorganisms of the same strain to form cellular aggregates. This property has been considered a desired feature of probiotics, since the biomass via auto-aggregation exerts beneficial effects (Collado et al., 2008; Krausova et al., 2019). As observed in Fig. 3.2, overall, the auto-aggregation abilities of *A. muciniphila* DSM 22959 and *L. rhamnosus GG* increased over time in both atmospheres. As depicted in Figure 3.2, both *A. muciniphila* DSM 22959 and *L. rhamnosus* GG exhibited similar auto-aggregation percentages when considering the same timepoint and atmospheric incubation condition ($p > 0.05$). In the same alignment, a previous study conducted by Cozzolino et al. (2020) reported auto-aggregation values at timepoints 2 and 24 h of 28.70 and 69.42% for *A. muciniphila* DSM 22959, and 15.19 and 65.21% for *L. rhamnosus* GG (Cozzolino et al. 2020). Furthermore, the slight discrepancies in the results reported by Cozzolino et al. (2020) study and those of present thesis may be attributed to different growth conditions and the protocol applied in the auto-aggregation test.

(a)

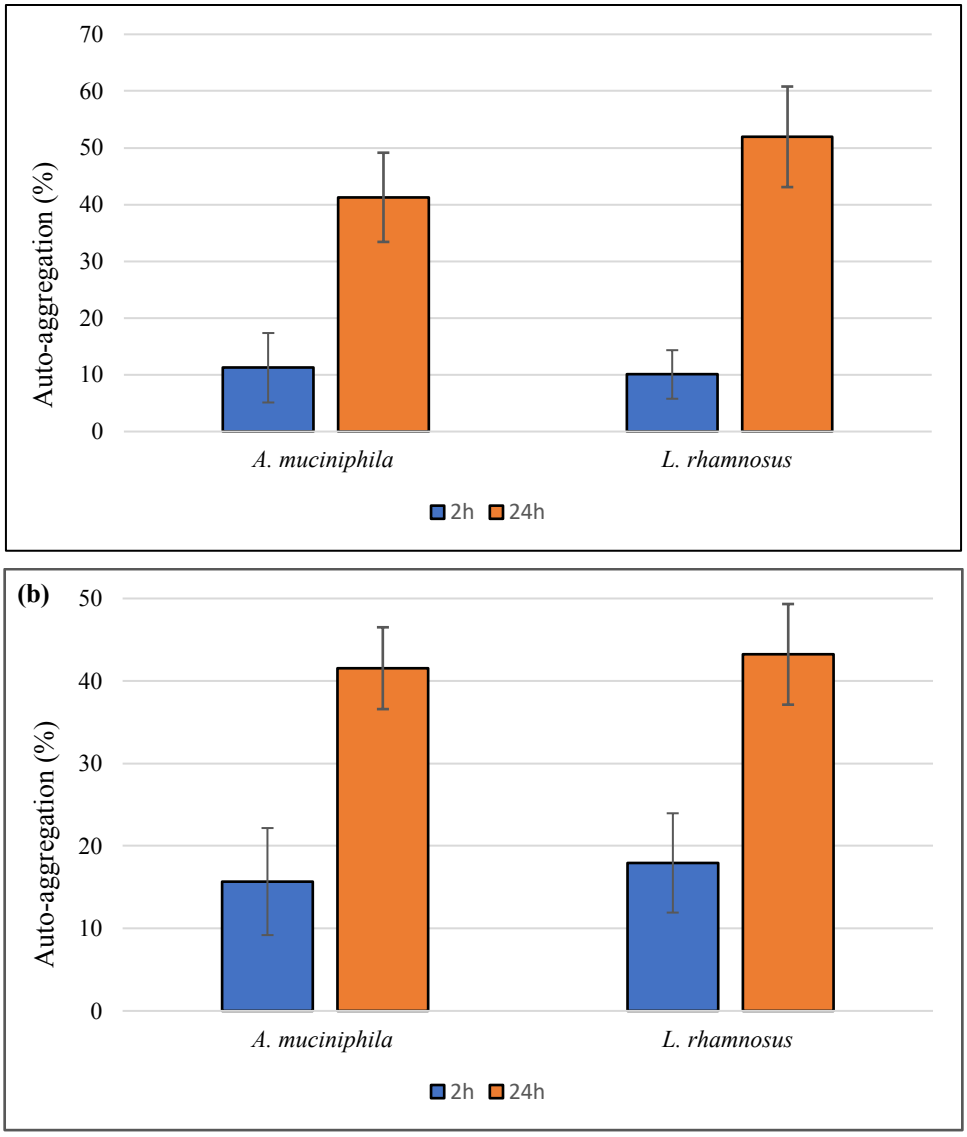

Figure 3.2 Auto-aggregation percentages of *L. rhamnosus* GG and *A. muciniphila* DSM 22959, after 2 hours (blue bars) and 24 hours (orange bars) of incubation at 37ºC under aerobic (a) and anaerobic (b) conditions. Error bars represent the standard deviation of the mean of at least 3 independent experiments. The symbol * indicates the statistically significant differences ($p < 0.05$) between each *A. muciniphila* DSM 22959 strain in comparison to the data obtained for *L. rhamnosus* GG at the same atmosphere condition and same incubation time.

Co-aggregation is described as the ability of aggregation between different strains, playing an important role in eliminating pathogens from the intestinal environment (Collado et al., 2008; Ferreira et al., 2011; Tuo et al., 2013). As shown in Table 3.1, *A. muciniphila* DSM 22959 and *L.rhamnosus* GG were able to co-aggregate with all pathogens at the tested time points at both atmospheres. Moreover, in general, co-aggregation abilities increased with increasing incubation periods, similarly to what was verified in the auto-aggregation assays. Additionally, our data showed that *A. muciniphila*

DSM 22959 and *L. rhamnosus GG* presented similar values of co-aggregation percentages in the same incubation conditions, since there were no statistical differences between co-aggregation values of both strains when considering the same atmosphere and incubation time ($p > 0.05$, Table 3.1).

Despite the existence of previous studies evaluating the co-aggregation abilities of *A. muciniphila* DSM 22959 and/or *L. rhamnosus* GG (Collado et al., 2008; Cozzolino, et al., 2020), the spectrum of pathogenic strains was different from that used in the present thesis, hindering potential comparisons. Furthermore, it has been shown that the co-aggregation percentages are strain-dependent (binomial probiotic/pathogen) and co-incubation time and conditions (Collado et al., 2008; Cozzolino et al., 2020; Ekmekci et al., 2009).

Table 3.1 Co-aggregation percentages of *A. muciniphila* DSM 22959 and *L. rhamnosus* GG with different pathogens after 2 and 24 h of incubation under aerobic and anaerobic conditions.

Pathogen	***Akkermansia muciniphila* DSM 22959**				***Lacticaseibacillus rhamnosus* GG**			
	Aerobiosis		**Anaerobiosis**		**Aerobiosis**		**Anaerobiosis**	
	2 h	**24 h**	**2 h**	**24 h**	**2 h**	**24 h**	**2 h**	**24 h**
S. intermedius ESB 2567	8.30± 4.11	35.06± 10.16	6.85 ± 3.91	36.63 ± 17.03	9.21 ± 6.78	43.70 ± 9.03	20.53 ± 15.43	36.57 ± 14.24
E. coli O157:H7	5.26± 4.36	33.59 ± 10.70	6.56 ± 7.44	27.37 ± 7.01	8.01 ± 6.92	44.39 ± 12.15	13.74 ± 7.43	31.23 ± 7.37
E. coli ATCC 25922	5.45± 0.92	38.41 ± 8.07	8.22 ± 5.82	29.42 ± 3.97	9.83 ± 2.89	43.74 ± 3.25	13.02 ± 6.88	26.46 ± 5.57
K. pneumoniae ESB	5.41± 3.68	33.59 ± 7.67	9.98 ± 3.79	29.36 ± 4.46	6.64 ± 3.80	38.67 ± 2.34	12.72 ± 8.22	28.53 ± 2.04
S. enterica ATCC 14028	5.06± 3.21	31.60 ± 8.53	8.48 ± 6.57	27.02 ± 2.63	7.45 ± 3.72	36.67 ± 6.15	15.00 ± 5.60	28.28 ± 2.33
Y. enterocolitica NCTC 10460	7.13± 3.93	26.99 ± 7.63	6.20 ± 3.67	27.13 ± 2.53	8.28 ± 5.51	38.71 ± 9.71	15.08 ± 7.36	29.31 ± 9.10
S. aureus DSM 11729	5.04± 4.50	36.66 ± 6.14	9.75 ± 7.73	32.71 ± 5.26	8.08 ± 6.21	48.32 ± 7.03	14.57 ± 6.50	37.80 ± 6.61
S. aureus ATCC 25923	4.69± 3.73	42.86 ± 4.25	9.85 ± 9.13	40.37 ± 1.78	8.48 ± 3.53	48.62 ± 5.03	17.76 ± 7.94	44.46 ± 7.74
L. monocytogenes NCTC 10357	15.08± 7.13	50.29 ± 7.71	15.30 ± 6.99	56.11 ± 1.35	15.40 ± 8.82	61.01 ± 5.04	22.56 ± 4.04	57.78 ± 5.11

3.3. Effect of probiotic *Akkermansia muciniphila* DSM 22959 against *Escherichia coli* damaged intestinal barrier

The intestinal epithelial barrier serves as a critical interface responsible for maintaining the proper functioning and balance of the intestinal microbiota (Bhat et al., 2020; West et al., 2015). In turn, any alteration in the composition of the intestinal microbiota, particularly an increase in bacteria triggering pro-inflammatory response, can have an impact on the barrier's function, leading to compromised or unstable conditions. This can contribute to the development of allergies, asthma, eczema, inflammatory diseases, and metabolic syndromes (Bhat et al., 2020; West et al., 2015). Consequently, probiotics have emerged as beneficial agents capable of combating external pathogens, neutralizing harmful toxins found in food, stimulating the production of mucus and other antimicrobial substances, and regulating both systemic and mucosal immune responses (Bhat et al., 2020; Zielińska et al., 2019). The goal of these probiotic actions is to promote and maintain the overall well-being and health of the host. Some studies have shown a correlation between adhesion ability and hydrophobicity in certain lactobacilli, indicating that the hydrophobicity of probiotics may contribute to their adhesion capacity (Collado et al., 2008; Kos et al., 2003; Tuo et al., 2013; Xu et al., 2009).

Caco-2 cells are a commonly used in vitro model to study intestinal epithelial cells. These cells are derived from human colon adenocarcinoma and possess several characteristics that make them very suitable for simulating the properties and functions of intestinal epithelium, including their simple handling for extended periods and their ability to form tight junctions while growing in vitro (Srinivasan et al., 2015). To investigate whether the probiotic bacterium *A. muciniphila* can enhance the integrity of the intestinal barrier in the presence of the pathogenic bacterium *E. coli*, competition, exclusion, and displacement tests were performed using the Caco-2 cell line. Thus, to measure the integrity of the intestinal barrier, TEER (Trans-Epithelial Electrical Resistance) and Phenol Red flux assays were employed.

Exclusion assays are employed to investigate whether probiotic bacteria possess the ability to exclude pathogenic bacteria from adhering to or colonising specific sites in the body (van Zyl et al., 2020). This is usually assessed by exposing target cells, in this case Caco-2 cells, to the probiotic strain followed by exposition to the pathogenic strain and then measuring the degree to which the probiotics prevent attachment or invasion by the

pathogen (Bhat et al., 2020). Competition assays assess the direct competition between probiotics and pathogenic bacteria, and by co-culturing these bacterial strains, it is possible to observe whether the presence of a probiotic strain influences the proliferation of the pathogen (Bhat et al., 2020; van Zyl et al., 2020). This way, it can be observed whether probiotics have a competitive advantage over pathogenic bacteria, reducing their harmful effects. Displacement assays assess the ability of probiotics to displace pathogenic bacteria that have already established colonisation in a particular location (van Zyl et al., 2020). These trials involve introducing probiotics into an environment where pathogenic bacteria are already present and assessing the probiotics' ability to compete with or displace pathogens from their established positions (Bhat et al., 2020). Thus, these tests provide insights into how probiotic bacteria may affect the growth and colonization of pathogenic bacteria in various environments, particularly in the gastrointestinal tract (van Zyl et al., 2020).

The quantitative assessment of the integrity of a barrier can be determined by measuring the Transepithelial Electrical Resistance (TEER) of a cellular monolayer, which is expressed in ohms (Srinivasan et al., 2015). More specifically, the more cohesive the epithelium is, the more resistance it will offer. On the other hand, Phenol Red Flux assays are commonly used to investigate the impact of probiotics and pathogens on the permeability of cell monolayers. Phenol red is added to the apical (upper) compartment, and the rate at which it crosses the epithelial barrier and accumulates in the basolateral (lower) compartment is determined by measuring the absorbance or fluorescence (Bhat et al., 2020). This assay relies on the principle that the permeability of the monolayer correlates with the movement of phenol red. So, if the epithelial barrier is compromised, there will be an increase in phenol red flux, indicating higher permeability. On the other hand, if the barrier remains intact, the flux will be lower, indicating lower permeability. Figure 3.3 illustrates the results obtained from the Phenol red flux assay and TEER measurement, providing insights on the effect of *A. muciniphila* on *E. coli* growth and colonization.

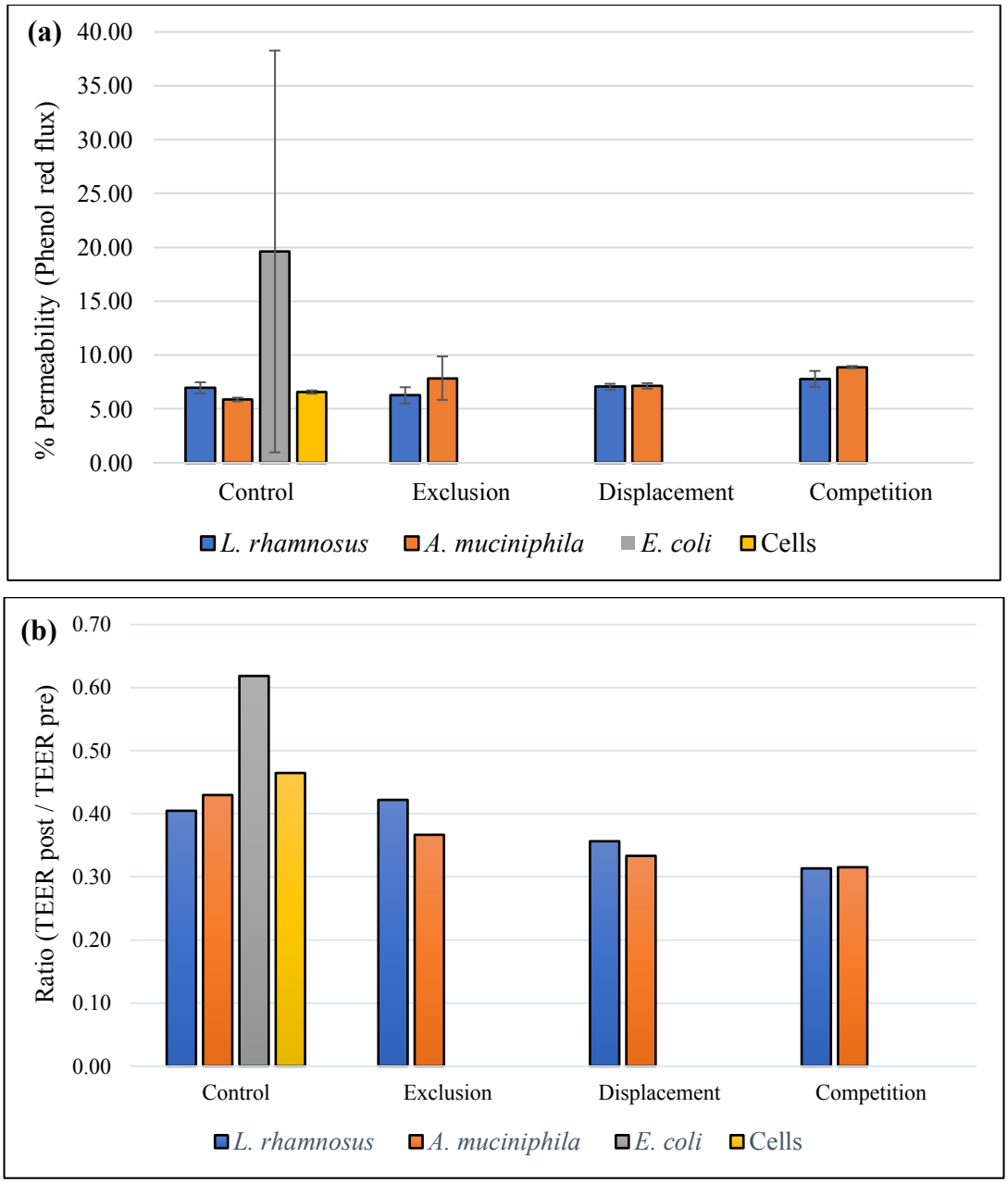

Figure 3.3 Effect of probiotic *A. muciniphila* and *L. rhamnosus* GG on *E. coli*-induced intestinal epithelial hyperpermeability in Caco-2 cell monolayer (cells) during exclusion, competition and displacement assays. (a) Phenol red diffusion assay and (b) TEER Measurement.

Analysing Figure 3.3 (a), it can be seen that exposing the Caco-2 cell layer to *E. coli* for 3 hours led to a non-significant ($p > 0.05$) increase in the flux of phenol red (19.62 ± 18.65%) compared to untreated control cells (6.57 ± 0.15%). Additionally, when the Caco-2 cell layer was treated solely with either the *A. muciniphila* or *L. rhamnosus* probiotic strains for the same duration, the permeability levels were nearly identical (5.88 ± 0.17% or 6.96 ± 0.52%, respectively) to those of the control cells ($p>0.05$). This indicates that the probiotic strains under study effectively do not impair the integrity of

the cellular junctions, thus, not causing any harm to the intestinal cells. Moreover, the presence of either *A. muciniphila* or *L. rhamnosus* did not significantly ($p > 0.05$) hinder the phenol red-induced intestinal hyperpermeability caused by *E. coli* during the exclusion (7.86 ± 2.02% or 6.26 ± 0.76%, respectively), displacement (7.14 ± 0.26% or 7.06 ± 0.28%, respectively), and competition (8.88 ± 0.11% or 7.79 ± 0.74%, respectively) experiments (Fig. 3.3 (a)). Regardless of the type of test conducted (exclusion, displacement, or competition), it seems that the percentage of permeability remains consistent. This suggests that the order in which the probiotic (*A. muciniphila* or *L. rhamnosus*) and the pathogen (*E. coli*) are added does not significantly affect the protective impact of the probiotic strains on the intestinal epithelium when *E. coli* is present.

In the competition test, where *A. muciniphila* and *E. coli*, as well as *L. rhamnosus* and *E. coli*, are added simultaneously, non-significant differences ($p > 0.05$) were reported, indicating that both *A. muciniphila* and *L. rhamnosus* are not effective in mitigating the disruptive effects of *E. coli*. Similarly, non-significant differences ($p > 0.05$) were observed in the exclusion test, demonstrating, once again, that both *A. muciniphila* and *L. rhamnosus* are not very efficient in reducing the disruptive effects of *E. coli*. Nevertheless, it is worth mentioning that in all tests, the protective effect of *A. muciniphila* was approximately 50% of the disruptive effect caused by *E. coli* alone (control). Thus, it is possible to infer that lower membrane integrity is achieved when *A. muciniphila* is subjected to competition with *E. coli* when compared to exclusion and displacement ($p < 0.05$) which indicates the inability of *A. muciniphila* to combat directly the effects caused by *E. coli* on membrane permeability.

In what concerns the transepithelial electrical resistance (TEER), Figure 3.3 (b) shows that exposing intestinal Caco-2 cells to *E. coli* for 6 hours resulted in a significantly higher $TEER_{post}/TEER_{pre}$ ratio (0.62 ± 1.25) when comparing with all the other tested conditions ($p<0.05$). This stands in contrast to the ratio values observed in control cells (0.46 ± 0.50) and cells treated with either probiotic *L rhamnosus* GG (0.40 ± 0.59) or probiotic *A. muciniphila* (0.43 ± 0.89) (Fig. 3.3 (b)). Also, this outcome was unexpected, when comparing to the results obtained with the phenol red flux assay, since the incubation with *E. coli* was indeed expected to cause a more severe decrease in the transepithelial resistance. However, for all the conditions tested and the respective controls (except for *E. coli* control), a similar trend to that previously observed with the phenol red flux assay is clear, since the transepithelial resistance seems to present no significant changes

between conditions. In addition, it is within the range of the controls subjected to the application of probiotic strains and only slightly decreased when comparing to the cells not exposed to any pathogenic or probiotic strains (Figure 3.3 (b)) Even so, due to the unexpected outcome of the *E. coli* control, the TEER analysis could not provide conclusive evidence regarding the resistance of the epithelial membrane and should be performed again, with optimized conditions. In fact, the untreated cells displayed a rather compromised epithelium (around 50 %, Figure 3.3 (b)), and thus, this assay does not provide sufficient grounds for drawing solid conclusions. This discrepancy could potentially be attributed to the need for methodological optimization, since the same plate was used for both the Phenol red flux and TEER assays, which might have affected the obtained results, by compromising the integrity of the cellular epithelium. Another possible explanation is that other assay conditions require optimization, as it is a lengthy procedure, involving multiple pipetting steps. These factors could potentially influence the viability of the cells, as they are no longer in their optimal environment and temperature (37º C) throughout the process. The cell differentiation time could also have been increased to 21 days in order to promote a better stability of the epithelium.

The potential of probiotic *Lactobacillus rhamnosus* (MTCC-5897) to counteract the impairment of intestinal barrier function caused by *Escherichia coli* ATCC 14948 was evaluated in a study conducted by Bhat et al. (2020). The study involved independent performance of Phenol red flux and TEER assays. Additionally, the incubation time between introducing the probiotic and the pathogen was set at 6 hours, which suggests the possibility of optimizing this duration in future trials (Bhat et al., 2020). In fact, in the present dissertation, the incubation period was set to 6 h in total (meaning 3h between each addition) due to limitations related to exposition of *A. muciniphila* to non-optimal conditions, observed in previous studies performed by our group (data not shown). The results obtained by Bhat et al. (2020) and colleagues showed that, intestinal cells exposed to *E. coli* significantly increased phenol red flux (6.73 ± 0.3% against 2.05 ± 0.2% in control) and decreased TEER (0.69 ± 0.01 against 1.05 ± 0.02 in control)), in contrast to control or *L. rhamnosus* treated cells.

Another study performed by Xu et al. (2009) evaluated the competitive inhibition of food-borne pathogens adhesion to Caco-2 cells by probiotic strains, one of which was *L. rhamnosus* GG. The results obtained demonstrated that the adhesion of *Listeria monocytogenes*, *Salmonella Typhimurium*, *Shigella boydii* and *Staphylococcus aureus* to Caco-2 cells was significantly inhibited by *L. rhamnosus* GG (Xu et al., 2009).

Therefore, it can be inferred that *A. muciniphila* exhibits the potential to promote the integrity of Caco-2 epithelium under conditions where cells are weakened by *E. coli* O157:H7. However, additional research is required to prove this advantageous characteristic.

3.4. Extrusion encapsulation

Considering the framework described in section 1.5, the next phase aimed to evaluate the viability and stability of *A. muciniphila* DSM 22959 entrapped into calcium-alginate capsules produced via extrusion during aerobic refrigerated storage and when exposed to *in vitro* gastrointestinal passage.

3.4.1. Encapsulation yield of calcium-alginate capsules entrapping *Akkermansia muciniphila*

The encapsulation yield (EY) was 60% (± 18%), calculated considering the initial (suspension used for extrusion; 2.10×10^{10} CFU) and final (calcium-alginate capsules; 1.26×10^{10} CFU) *A. muciniphila* viable cell numbers. This value indicates that this encapsulation technique is suitable for *A. muciniphila* entrapment since the order of magnitude of cell density (10^{10} CFU per gram of capsule) was maintained.

Comparing the obtained EY with other studies using similar encapsulation techniques but different probiotic strains, it falls within the reported range. For example, a study by (Amine et al., 2014) achieved EY values ranging from 31% to 65% for encapsulating *Bifidobacterium longum* ATCC 15708 using sodium alginate and/or O-palmitoylated alginate at various concentrations. Additionally, Frakolaki et al. (2021) implemented an encapsulation strategy for *Bifidobacterium animalis* subsp. *lactis* BB-12® via extrusion using alginate alone or in combination with other encapsulating agents such as inulin, glycerol, glycose and L-cysteine-HCl and obtained EY values ranging from 58.6% to 100% depending on the encapsulation conditions and materials used. In the case of *A. muciniphila* encapsulation, van der Ark et al. (2017) reported an EY of 97.5% using a water-in-oil-in-water double emulsion technique. However, the encapsulated *A. muciniphila* showed reduced viability after 4 days of refrigerated storage either under aerobic or anaerobic conditions. Afterward, Marcial-Coba et al (2018) encapsulated *A. muciniphila* DSM 22959 in a xanthan/gellan gum matrix, via the extrusion method, with

a subsequent freeze-drying step, reporting an encapsulation efficiency ranging from 12 to 76%, depending on the cryoprotective agents employed. Recently, Almeida and collaborators (2022) immobilized *A. muciniphila* DSM 22959, with a 64.4% entrapment efficacy, in a dual hydrocolloid matrix of alginate and denatured whey protein isolate by the emulsification/internal gelation method. While the wide variety of encapsulating materials and techniques may explain the distinct values obtained in this work and in those described in the literature, our results suggest that the extrusion technique in calcium-alginate matrix seems be an efficient technological strategy for entrapment of *A. muciniphila*.

3.4.2. Viability of *Akkermansia muciniphila* DSM 22959 upon extrusion encapsulation

Although there is no consensus regarding the minimum effective probiotic dose, it is usually accepted that probiotic products should have a minimum concentration of 10^6 CFU per mL or per gram and that a total of 10^8 - 10^9 probiotic microorganisms should be consumed daily to elicit health benefits (Kechagia et al., 2013). For this reason, one of the major aspects when developing probiotic formulations refers to the ability of these to ensure the maintenance of probiotic viability throughout manufacturing procedure, chain distribution until it reaches the consumer (Barbosa et al., 2022). Additionally, in literature the refrigeration temperature of 4ºC has been related with high viability level of *A. muciniphila*, either in free or encapsulated forms (Almeida et al., 2022; Barbosa et al., 2022; Marcial-Coba et al., 2018, 2019). Taking this into account, free and encapsulated *A. muciniphila* were stored at 4ºC under aerobic conditions and their viability was assayed, at specific timepoints, for 28 days. As it can be observed in Figure 3.4, *A. muciniphila* exhibited a high stability in viability (loss < 0.2 log-cycle) after 28 days of refrigerated aerobic storage, maintaining its viability at around 8 log CFU/g. In contrast, free cell numbers decreased approximately 1 log cycle. Notably, the present results concerning encapsulated *A. muciniphila* viability and stability throughout refrigerated aerobic storage contrast positively with those reported in literature up to the present moment. In fact, van der Ark et al. (2017) reported a sharp viability reduction to viable cell numbers below 0.01 CFU/mL for *A. muciniphila* encapsulated in water-in-oil-in-water double emulsion when stored at 4ºC for 72 h in both atmospheric conditions: anaerobiosis and aerobiosis. Later, Marcial-Coba et al. (2018) evaluated the viability of

A. muciniphila encapsulated via extrusion in a xanthan and gellan gum matrix with subsequent freeze-drying, throughout 30 days in both aerobic and anaerobic storage conditions at temperatures of 4°C and 25°C. These researchers reported significant decrease of at least 2 log cycles in viability of the freeze-dried microencapsulated *A. muciniphila* after 30 days of storage under both anaerobic and aerobic conditions, at both 4°C and 25°C, when compared with initial concentration (Marcial-Coba et al., 2018). Chang and colleagues encapsulated *A. muciniphila* in a matrix of succinate-grafted alginate doped with epigallocatechin-3-gallate via spray-drying and the authors observed a protective effect of the matrix on the bacterial viability when comparing with the free cells but this was performed during a storage period of only 12 days and under anaerobic, refrigerated (4ºC) conditions (Chang et al., 2020). Another investigation, conducted by Barbosa et al. (2022) explored spray-drying encapsulation technique with different dairy-based matrices to enhance the viability of *A. muciniphila* over aerobic storage. The results indicated that the viability remained around 10^7 CFU/g up to 28 days at 4ºC under aerobic conditions, using 10% skim milk powder matrix. (Barbosa et al., 2022). More recently, Almeida et al. (2022) encapsulated *A. muciniphila* in a dual hydrocolloid matrix containing alginate and denatured whey protein isolate by emulsification/internal gelation and they assessed viability of encapsulated bacteria and free counterpart over 95 days of refrigerated storage under aerobic and anaerobic conditions. These researchers reported that during the initial 30 days of refrigerated storage, there was a similar reduction in viability of both free and encapsulated cells. However, after 95 days of storage, the viability of encapsulated *A. muciniphila* experienced a sharper decrease in both atmospheres (Almeida et al., 2022). Thus, the results obtained within the present thesis surpass the findings obtained in previous works, since the applied extrusion protocol is a simple, low-cost and well-established encapsulation technique that allowed to maintain the viability of encapsulated *A. muciniphila* cells at levels higher than 10^8 CFU/g after 28-days under feasible household storage conditions, namely aerobic storage at 4ºC. However, the process is relatively time-consuming, and the produced capsules are relatively large, which are pointed as the main drawbacks of this technique (Koh et al., 2022; Machado et al., 2020).

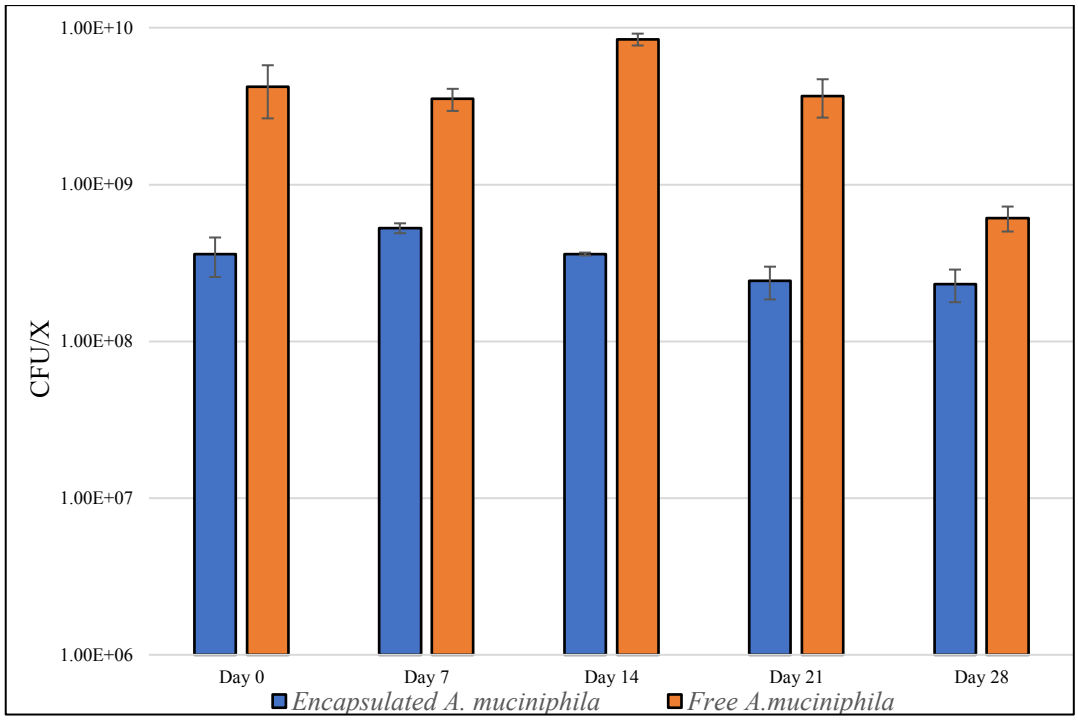

Figure 3.4 Viability of *A. muciniphila* DSM 22959 free (CFU/mL) and entrapped (CFU/g) in calcium-alginate capsules produced via extrusion during aerobic refrigerated storage at 4ºC.

3.4.3. Survival of *Akkermansia muciniphila* entrapped in alginate capsules when exposed to simulated gastrointestinal passage

It has been postulated that to elicit its beneficial effect, a probiotic microorganism must reach the target site, in adequate viable cell numbers (Hill et al., 2014). Therefore, it is important that delivery systems enclosing probiotic strains must be resistant to the adverse gastrointestinal conditions, allowing to deliver the probiotic strain in the optimal required conditions to the target site, to trigger the expected benefits. In the present study, the survival of free and encapsulated *A. muciniphila* cells was assessed under simulated gastrointestinal conditions in the following timepoints: 1 and 28 days of refrigerated aerobic storage and the results are presented in Table 3.2.

Table 3.2 Evolution of viable cell numbers of *A. muciniphila* DSM 22959 in free form (CFU/mL) or when entrapped in capsules (CFU/g) during *in vitro* gastrointestinal passage at 1 and 28 days.

	CFU/X ± S.D.			
	Day 1		Day 28	
	Free cells *A. muciniphila* DSM 22959	Capsules with *A. muciniphila* DSM 22959 cells	Free cells *A. muciniphila* DSM 22959	Capsules with *A. muciniphila* DSM 22959 cells
Initial phase	$3.40 \pm 0.39 \times 10^9$	$3.82 \pm 1.26 \times 10^8$	$6.12 \pm 1.11 \times 10^8$	$2.32 \pm 0.55 \times 10^8$
Gastric phase	$2.57 \pm 0.31 \times 10^{10}$	$2.74 \pm 1.17 \times 10^8$	$7.46 \pm 6.91 \times 10^6$	$1.15 \pm 0.12 \times 10^8$
Intestinal phase	$4.83 \pm 0.36 \times 10^9$	$1.57 \pm 0.33 \times 10^8$	$< 8 \times 10^5$	$2.93 \pm 1.09 \times 10^7$

As presented in table 3.2, on day 1 of storage both free and encapsulated *A. muciniphila* maintained viability throughout *in vitro* gastrointestinal passage, observing, a maintenance of magnitude order in viability of 10^9 CFU/mL and 10^8 CFU/g, respectively. In fact, in the literature it has been reported that *A. muciniphila* in free form exhibits a good resilience when exposed to gastrointestinal conditions (Almeida et al., 2022a; Machado et al., 2020). This natural resilience observed at early stage (at day 1) may be explained by the presence of an acid resistance system in *A. muciniphila* cells and possibly to reduced activity of bile salts against Gram-negative bacteria (Begley et al., 2005; Petschacher & Nidetzky, 2016).

Regarding the 28 days of storage, when exposed to the *in vitro* digestion protocol, the encapsulated bacteria suffered a reduction in viable cell numbers around 1-log cycle (achieving a final viability level around 10^7 CFU/g), while the free cells recorded a viability reduction of more than 2.5-log cycles (final viability level lower than 8×10^5 CFU/mL). These results showed a higher stability for the encapsulated bacteria throughout gastrointestinal passage than their free counterpart at timepoint 28 days of storage. In this alignment, a study conducted by Almeida et al. (2022) demonstrated that as storage time increased, *A. muciniphila* encapsulated in an alginate: denatured whey protein isolate matrix via emulsification/internal gelation showed higher stability when exposed to gastrointestinal passage than its free counterpart. Specifically, these researchers recorded viability reductions in free and encapsulated *A. muciniphila* in *ca.* 2 and lower than 1 log cycle, respectively, at timepoint of 30 days of refrigerated aerobic storage (Almeida et al., 2022). Still in this context, Barbosa and coworkers demonstrated that encapsulation of *A. muciniphila* via spray-drying in 10% skim milk using inlet/outlet

temperatures of 150/65ºC mitigated the detrimental effects of extended refrigerated aerobic storage for up 60 days with subsequent gastrointestinal passage allowing a probiotic survival at levels of at least 10^7 CFU/g (Barbosa et al., 2022).

Thus, the extrusion procedure presented herein seems to be a promising strategy to deliver *A. muciniphila* offering a protective effect during gastrointestinal transit, even after prolonged refrigerated aerobic storage. Concurrently, this encapsulation technique ensures the delivery of *A. muciniphila* at adequate levels in order to fulfil the criteria recommended for a probiotic product (minimum threshold of 10^6 CFU/mL or CFU/g).

3.5. Cheese spread incorporating *Akkermansia muciniphila* DSM 22959

In the last phase of the present thesis, it was developed a cheese spread incorporating *A. muciniphila* based on "Requeijão" (77% m/m) and Greek-style yogurt (23% m/m). Subsequently, this innovative food was characterized in terms of microbiological and physicochemical parameters, total phenolic content and antioxidant, antidiabetic and antihypertensive activities, as well as its protective effect on *A. muciniphila* viability during simulated gastrointestinal passage.

3.5.1. Microbiological parameters of cheese spread

One of the major concerns among the food industry and the community is the contamination of food by spoilage microorganisms and environmental pathogens. Therefore, microbiological quality is a crucial aspect when it comes to cheese or derived products production (Fusco et al., 2020). In the literature, whey cheese matrices are described as rich in nutrients in readily available form which, combined with their high pH, high moisture content, low salt concentration and water activity near to 1, may enhance the growth of spoilage microorganisms (Hough et al., 1999; Madureira et al., 2011). To address this issue, thermal treatments have been suggested as valuable strategies to prevent microbial contamination and ensure the microbiological quality and safety of the final products (Soni et al., 2021). Taking this into consideration, a thermal treatment (90 ºC for 10 minutes in water bath) was applied to cheese spread before incorporating *A. muciniphila* to prevent microbial contamination during 21 days of refrigerated aerobic storage. The microbiological analysis showed that microbial counts

of lactic acid bacteria, aerobic mesophilic microorganisms, fungi and Enterobacteriaceae in cheese spread incorporating *A. muciniphila* were below the limit of detection of the CFU plating technique, i.e., lower than 1000 CFU/g, in all sampling timepoints (0, 7, 14, and 21 days after refrigerated storage). Thus, these results indicate that this probiotic cheese spread had a shelf-life of at least 21 days under refrigerated storage conditions, in accordance with the previous findings of Faustino and colleagues (Faustino et al., 2023). The viability of probiotics in food products is another crucial factor. Factors such as pH, temperature, and processing techniques could impact the viability of the probiotic during manufacturing and subsequent storage (Terpou et al., 2019). Taking this into consideration, the viability of *A. muciniphila* was evaluated throughout the refrigerated aerobic storage for 21 days. As it can be observed in Figure 3.5, *A. muciniphila* DSM 22959 was successfully incorporated into the dairy matrix, the viable cell numbers in the cheese spread and free forms were similar at the day of cheesemaking, i.e., around10^9 CFU/g and around 10^9 CFU/mL, respectively. After an aerobic storage period of 7 days at 4°C, both the free cells and the cheese incorporated with *A. muciniphila* exhibited an increase in viability. The observed increase in the survival rate may be attributed to the recovery of previously damaged cells during the production and incorporation of the bacteria into the cheese, such as centrifugation. Specifically, in the case of *A. muciniphila*, which possesses relatively small cells (0.6-1.0 μm according to Derrien et al. (2004)), higher centrifugation speeds (12.000 x g as reported by Barbosa et al., (2022)) were necessary to obtain the maximum cell yield. After 14 days of storage, a slight decrease in *A. muciniphila* cell viability was observed independently of its form –incorporated in the cheese spread or as free cells. At the end of storage (21 days), the viability of *A. muciniphila* incorporated in cheese spread and in free form was around 6.57 x 10^8 CFU/g and 5.78 x 10^8 CFU/mL, respectively (Figure 3.5). In fact, the *A. muciniphila* viability loss either in cheese or free form was lower than 0.5 log cycle in relation to day 0. Furthermore, these results suggest that cheese spread could be an excellent food matrix to safeguard viability and stability of *A. muciniphila* during refrigerated aerobic storage. In fact, *A. muciniphila* viability was maintained at acceptable levels during storage, since it is generally accepted that probiotic products should have a minimum concentration of 10^6 CFU/mL or gram (Kechagia et al., 2013). In literature, it has been described that the incorporation of probiotic bacteria has been successfully performed in whey cheese matrices. Several strains of *Lactobacillus acidophilus*, *Lacticaseibacillus casei* (formerly *Lactobacillus casei*), *Lacticaseibacillus paracasei* (formerly *Lactobacillus paracasei*),

Lacticaseibacillus rhamnosus (formerly *Lactobacillus rhamnosus*), *Levilactobacillus brevis* (formerly *Lactobacillus brevis*) and *Bifidobacterium animalis* were able to maintain viability levels above 10^7 CFU/g during at least 21 days of refrigerated storage in whey cheeses with or without additives (Garcia et al., 2022; Madureira et al., 2006b, 2008, 2015).

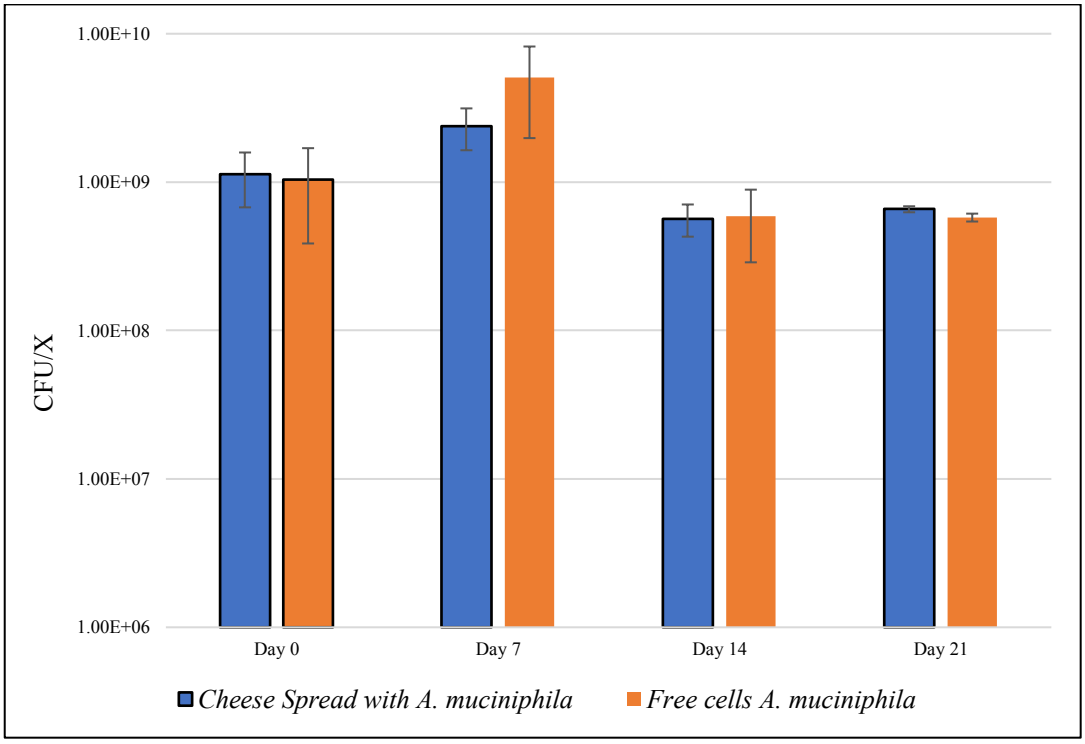

Figure 3.5 *Akkermansia muciniphila* viable cell numbers incorporated in cheese spread (expressed in CFU/g) and free cells (expressed in CFU/mL) and their evolution throughout refrigerated (4ºC) aerobic storage for 21 days.

3.5.2. Physicochemical characteristics of cheese spread

The physicochemical characteristics of cheese spread with and without incorporation of *A. muciniphila* are presented in Table 3.2. Overall, the incorporation of *A. muciniphila* into cheese spread did not alter significantly the texture, water activity, and pH values when compared with those of the control cheese i.e., without *A. muciniphila* ($p>0.05$). Furthermore, the color difference (ΔE) between cheese spread with *A. muciniphila* and cheese spread control was below the minimum detection limit- ($\Delta E = 0.771 < 1$), hence the two types of cheese spread were not distinguishable by the human eye.

Thus, it is possible to conclude that, at day 1 of storage, there are no significant physicochemical alterations in the cheese spread incorporating *A. muciniphila* when

compared with the control counterpart. These observations are desirable because they may indicate a potentially high acceptability of this probiotic cheese spread by the consumers (Madureira et al., 2015). However, these analyses were performed only at day 1 of storage, so in order to better understand if the *A. muciniphila* along the time of storage altered the physicochemical characteristics of the cheese spread, it would be of great importance to perform the same analyses along the storage period.

Table 3.3 Physicochemical characteristics of cheese spread with and without A. *muciniphila.*

	Cheese spread with *A. muciniphila*	**Cheese spread without *A. muciniphila* (control)**
Texture		
Firmness (g)	692.03 ±10.61	919.20 ±138.93
Consistency (g/s)	12329.29 ± 877.00	14717.56 ± 2516.96
Cohesiveness (g)	-469.89 ± 64.92	-615.96 ± 77.90
Work of cohesion (g/s)	-646.66 ± 162.82	-787.33 ± 179.99
Color		
L	77.68 ± 0.67	76.97 ± 1.52
a	1.95 ± 0.06	1.77 ± 0.03
b	3.41 ± 0.31	3.62 ± 0.03
Water activity	0.966 ± 0.005	0.968 ± 0.003
pH	6.00 ± 0.04	5.98 ± 0.01

The symbol * marks the statistically significant differences between cheese spread with *A. muciniphila* and control cheese spread regarding each specific physicochemical parameter.

3.5.3. Total phenolic content and antioxidant activity of cheese spread

The main dietary sources of polyphenols are wine, beer, coffee, tea, and plant-based foods (Nardini, 2022). Phenolic compounds are substances produced during metabolism that consist of multiple phenol groups. These compounds are very active in neutralizing free radicals, demonstrate antioxidant properties, and serve as agents with antitumor, antimicrobial, and antimutagenic effects (Khan et al., 2018; Petti & Scully, 2009; Shui & Leong, 2002). It is important to note that the total content of phenols can vary depending

on several factors, including the food matrix, the nature of the process, and the period of treatment (Arfaoui, 2021; D'Archivio et al., 2010)

As it can be observed in Table 3.4, the total phenolic content present in cheese spread with *A. muciniphila* and in control were low, showing concentrations ranging from 0.36 and 0.38 mg gallic acid equivalents/ g for dried cheese spread with *A. muciniphila* and cheese spread control, respectively. In literature, the phenolic compounds content and antioxidant activity have been correlated in direct proportionality, i.e., higher levels of phenolic compounds have been linked to high antioxidant activity (Khan et al., 2018; Song et al., 2010). Thus, as expected given the low concentration of phenolic compounds in both cheese spreads, the antioxidant activity was below the limit of detection of the ABTS assay (lower than 0.0625 µmol of Trolox equivalents/g of dried cheese spread). Even so, it would be useful to carry out a qualitative analysis to understand exactly which phenolic compounds are present in the matrix, even if in minor quantities (Khoddami et al., 2013). This analysis could be performed by spectrophotometric or chromatographic techniques (Khoddami et al., 2013).

Table 3.4 Total phenolic content and antioxidant activity measured using ABTS assay, of cheese spread incorporating *A. muciniphila* and control.

	Cheese spread with *A. muciniphila*	**Cheese spread without *A. muciniphila* (control)**
Total phenolic content (in mg gallic acid equivalents/ g of dried cheese spread)	0.36 ± 0.04	0.38 ±0.02
ABTS scavenging activity (in µmol of Trolox equivalents/g of dried cheese spread)	< 0.0625	< 0.0625

Note: The symbol * indicates the statistically significant differences ($p < 0.05$) between cheese spread incorporating *A. muciniphila* and cheese control in each parameter: total phenolic content and ABTS scavenging activity.

Since phenolic compounds are a type of secondary metabolite that is mainly synthesized by plants (Lin et al., 2016), it would be expected that this cheese spread would have a low content of them, since it is a product of animal origin. It is known that phenolic compounds present in cow's milk, such as phenols (thiophenol, cresols, ethyl phenols),

hydroxybenzoic acids, hydroxycinnamic acids, flavonoids, and anthocyanidins, are derived from the feed, although they may also result from amino acid conversion (O'Connell & Fox, 2001). Furthermore, there are no statistically significant differences in total phenolic content and ABTS scavenging activity between the control cheese and the cheese spread incorporating *A. muciniphila* ($p > 0.05$), which suggests that *A. muciniphila* does not influence the phenolic compounds amount present in the cheese spread. Since this activity was determined on the day of production (day 0), this result was expected. To evaluate if *A. muciniphila* presents some kind of metabolic activity, it should have been performed the activity of total phenolic compounds throughout or at the end of the storage. Moreover, it is important to note that these results are in accordance with Faustino et al. (2023) study that reported a low ABTS scavenging activity (ABTS radical scavenging percentage around 10%) in a cheese spread with similar composition to that produced in this thesis, namely: containing 75% (m/m) of "Requeijão" and 22% (m/m) of Greek-style yoghurt.

3.5.4. Antidiabetic activity of cheese spread

According to the World Health Organization, diabetes "is a chronic, metabolic disease characterized by elevated levels of blood glucose (or blood sugar), which leads over time to serious damage to the heart, blood vessels, eyes, kidneys and nerves" (World and Health Organization, 2023). Worldwide, diabetes was responsible for 6.7 million deaths in 2021 and 537 million adults (20-79 years) are living with diabetes (International Diabetes Federation, 2022). Previous meta-analyses showed an inverse association between consumption of yogurts and cheese and risk of type-2 diabetes (Aune et al., 2013; Gao et al., 2013). Taking this into consideration, the antidiabetic potential of cheese spread with and without *A. muciniphila* (control) were assayed, determining the α-glucosidase inhibitory activity.

As it can be observed in Figure 3.6, both cheese spreads (with *A. muciniphila* and control) displayed α-glucosidase inhibitory percentage around 100% and no statistically significant differences in α-glucosidase inhibitory activity were found between these cheeses ($p > 0.05$). This finding suggests that the addition of a probiotic strain such as *A. muciniphila* does not impact the α-glucosidase inhibitory activity either positively or negatively. It is important to note that in vivo studies highlight the potential of *A. muciniphila* in reducing the accumulation of body mass and the levels of plasma TNF-α.

Furthermore, it has been observed to enhance the number of goblet cells, promote mucin secretion, and restore the integrity of the gut barrier (Deng Si-si et al., 2022). Collectively, these effects play a role in improving symptoms associated with diabetes. The assay performed on antidiabetic activity does not specifically target insulin sensitivity but instead assesses the inhibition of α-glucosidase. As a result, it is not possible to establish a solid correlation between *A. muciniphila* incorporation into cheese spread matrix and increased antidiabetic potential, as two different parameters are being evaluated. Although the addition of *A. muciniphila* did not impact the inhibitory activity of α-glucosidase in the cheese matrices, the inclusion of this probiotic in the cheese spread may still hold value, as the bacterium may exert antidiabetic effects in the human body after consumption. Therefore, to validate this hypothesis, further in vivo studies targeting the antidiabetic potential of cheese spread incorporating *A. muciniphila* should be carried out. Furthermore, the high antidiabetic activity of cheese spread (*ca.* 100%) detected in the present work differ from the findings obtained by Faustino et al. (2023). In fact, these researchers reported α-glucosidase inhibitory activity below 2% for cheese spread composed by 75% (m/m) whey cheese and 22% (m/m) of Greek-style yogurt (Faustino et al., 2023). Possible explanation for these discrepancies may be attributed to different protocols applied for preparation of extract that were used in the antidiabetic activity assay.

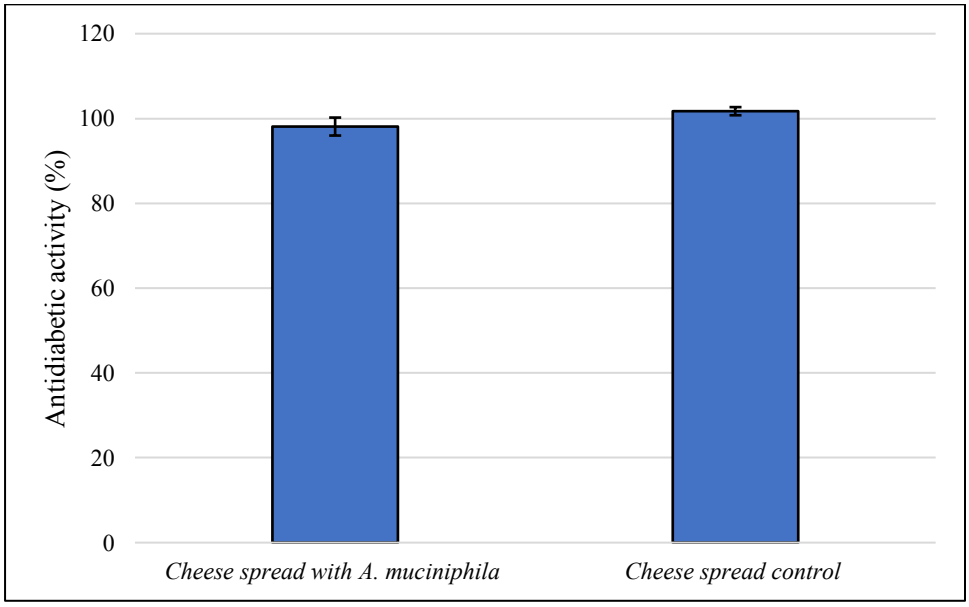

Figure 3.6 Antidiabetic activity (%) of cheese spread incorporating *A. muciniphila* DSM 22959 and cheese control upon production (day 1).

3.5.5. Antihypertensive activity of cheese spread

Hypertension, commonly referred to as high blood pressure, is a major cause of premature death worldwide (World and Health Organization, 2023). Notably, the intake of dairy products has been associated with reduced risk of hypertension (Heidari et al., 2021). Taking this into consideration, the antihypertensive activity of cheese spread with and without (control) *A. muciniphila* was determined using the ACE inhibition assay. As it can be seen in Figure 3.7, the ACE inhibition percentage was 49.18% (± 25.54) and 41.35% (± 11.18) for cheese spread incorporating *A. muciniphila* and control, respectively. Furthermore, there are no statistically significant differences in the antihypertensive activity between the cheese spread with *A. muciniphila* and the control sample ($p > 0.05$). Thus, these results suggest that *A. muciniphila* incorporation does not affect the antihypertensive effect of the cheese spread properties (measured in terms of ACE inhibition percentages). Additionally, as this activity was only performed on the day of production, it is only possible to infer that the antihypertensive properties present are due to the matrix itself. To prove this hypothesis, an in vivo study targeting the antihypertensive activity of the cheese spread incorporating *A. muciniphila* must be performed. Moreover, the present thesis reports higher ACE inhibition percentages for cheese spread than the values recorded in the study of Faustino et al. (2023). Indeed, these researchers reported undetectable ACE inhibition levels for cheese spread containing 75% (m/m) whey cheese and 22% (m/m) of Greek-style yogurt (Faustino et al., 2023). Possible explanation for these disagreements may be attributed to the same reason appointed for the antidiabetic activity namely different protocols applied for extract preparation.

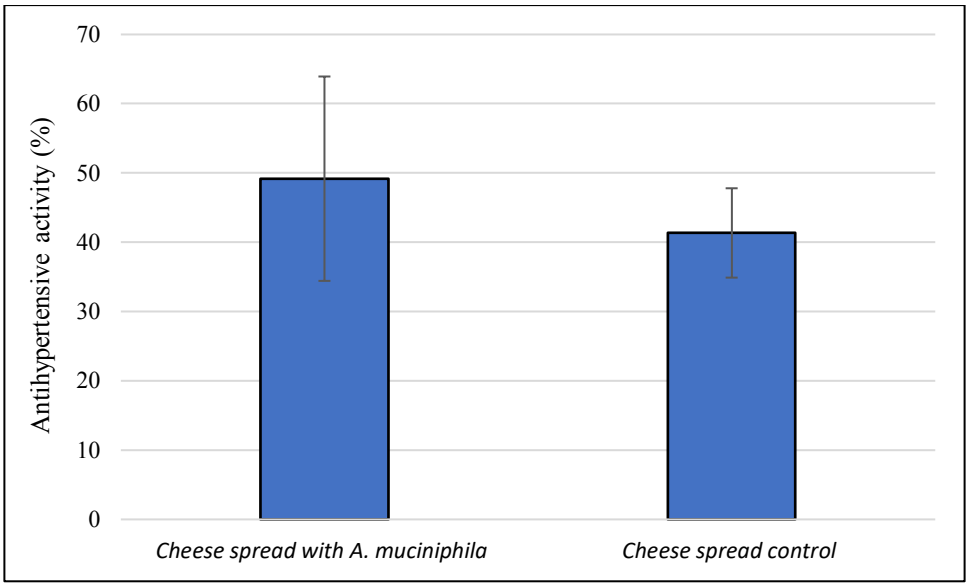

Figure 3.7 Antihypertensive activity (%) of cheese spread incorporating *A. muciniphila* DSM 22959 and cheese control upon production (day 1).

3.5.6. Survival of *Akkermansia muciniphila* incorporated in cheese spread and as free cells when exposed to simulated gastrointestinal passage

As previously mentioned, the probiotic cells must survive the harsh gastrointestinal conditions, reaching the intestine in adequate viability level, in order to exert beneficial effects (Hill et al., 2014). Taking this into consideration, cheese spread incorporating *A. muciniphila* and its free counterpart after 1 and 21 days of refrigerated aerobic storage were exposed to simulated gastrointestinal passage, with the aim to investigate the potential protective effect of the dairy matrix (cheese spread) on *A. muciniphila* viability throughout gastrointestinal transit. As can be observed in Table 3.5, in both timepoints (day 1 and 21), *A. muciniphila* cells delivered via cheese spread incorporation maintained their viability throughout gastrointestinal passage. In opposite, free cells suffered viability reductions of at least 2 log cycles when exposed to *in vitro* digestion protocol, at 1 and 21-days of refrigerated aerobic storage. These results suggest that the cheese spread matrix offers protection toward *A. muciniphila* viability during gastrointestinal passage, even after prolonged refrigerated aerobic storage for 21 days.

Furthermore, these results are in agreement with previous studies demonstrating that cheese matrices offer a high protection to probiotic microorganisms throughout gastrointestinal transit (Pitino et al., 2012; Stanton et al., 1998). This protective effect

may be attributed to the cheese spread´s unique traits such as availability of nutrients, fat and proteins that form a solid matrix with high buffering capacity and low oxygen content that safeguard probiotic microbial cells during the passage through stomach to the intestine (Rolim et al., 2020).

Table 3.5 Evolution of viable cell numbers of *A. muciniphila* DSM 22959 in free form (CFU/mL) or when incorporated in cheese spread (CFU/g) during *in vitro* gastrointestinal passage at 1 and 21 days.

	CFU/X ± S.D.			
	Day 1		Day 21	
	Free cells *A. muciniphila* DSM 22959	*A. muciniphila* DSM 22959 cells incorporated in cheese spread	Free cells *A. muciniphila* DSM 22959	*A. muciniphila* DSM 22959 cells incorporated in cheese spread
Initial phase	$2.00 \pm 0.14 \times 10^9$	$1.13 \pm 0.003 \times 10^9$	$5.78 \pm 0.35 \times 10^8$	$6.57 \pm 0.31 \times 10^8$
Gastric phase	$5.50 \pm 1.27 \times 10^6$	$9.60 \pm 0.75 \times 10^8$	$9.20 \pm 3.96 \times 10^6$	$1.86 \pm 0.71 \times 10^9$
Intestinal phase	$< 8 \times 10^5$	$2.05 \pm 0.15 \times 10^9$	$1.95 \pm 1.62 \times 10^6$	$6.47 \pm 3.56 \times 10^8$

4. Conclusion

In recent years, the next generation probiotic *A. muciniphila* has been recognized as a keystone species for promotion of gut health and consequently its incorporation in new pharmaceutical formulations and food products has been proposed. However, there is a lack of effective delivery systems able to ensure its high probiotic stability and viability during manufacturing, product shelf-life and after consumption, namely, throughout gastrointestinal transit. In this thesis, strategies based on encapsulation via extrusion in a calcium alginate matrix and a dairy food vector, comprising "Requeijão" and Greek-style yogurt, were explored successfully as protective approaches to increase viability and stability of *A. muciniphila* throughout refrigerated aerobic storage and when exposed to harsh gastrointestinal conditions.

In the first phase, the phenotypic characterization of *A. muciniphila* DSM 22959 regarding certain probiotic properties showed that this strain displayed a low cell-surface hydrophobicity, but it exhibited an ability for auto-aggregation and co-aggregation with pathogens in a similar extent to that verified for *L. rhamnosus* GG (known as a widely used and well-characterized probiotic strain). The ability of *A. muciniphila* to maintain or improve the intestinal barrier integrity in the presence of pathogens still requires further studies, however, the preliminary assays conducted herein show a tendency for a behavior similar to that of *L. rhamnosus* GG in conferring protection against the deleterious effects of *E. coli* in the conditions tested.

In the second phase, encapsulation via extrusion and immobilization in a dairy food vector were addressed as technological strategies to enhance viability and stability of *A. muciniphila* during refrigerated aerobic storage and gastrointestinal transit. Overall, the extrusion in a calcium alginate matrix ensured large production yields (around 60% encapsulation efficiency) with high-loaded capsules that maintained a cell density in the order of magnitude of 10^8 CFU/g throughout 28 days of refrigerated aerobic storage. Moreover, as storage time increased, encapsulated *A. muciniphila* demonstrated higher survival rates and stability when subjected to in vitro gastrointestinal conditions than its free counterpart. In turn, the incorporation of *A muciniphila* in a dairy matrix containing 77% (m/m) of "Requeijão" and 23% (m/m) of Greek-style yogurt led to the development of an innovative food, a cheese spread product, with high microbiological quality and interesting biological activities, namely antidiabetic and antihypertensive properties. Concurrently, this novel food ensured a high *A. muciniphila* viability level (> 10^8 CFU/g)

after refrigerated aerobic storage for 21 days and when subsequently exposed to simulated gastrointestinal conditions. Advantageously, the cheese spread incorporating *A. muciniphila* exhibited a similar profile in terms of texture, color, water activity and pH, when compared with its control, suggesting a potentially high acceptability among consumers.

5. Future work

The results presented in this thesis provided valuable information on some probiotic properties of *A. muciniphila* and exhibited the first insights regarding encapsulation via extrusion in calcium alginate matrix and incorporation in a cheese spread containing whey cheese and Greek-style yogurt, as technological strategies to increase *A. muciniphila* viability and stability during refrigerated aerobic storage and during its passage through simulated gastrointestinal conditions. However, it also raised some questions that should be addressed in future research works.

Given the various physiological traits associated with probiotic properties, there is a pressing need to further investigate additional characteristics of *A. muciniphila* DSM 22959 underlying its health beneficial effects. Specifically, an in-depth analysis addressing the possible mechanisms involved in its adhesion to intestinal epithelium such as identification of specific adhesins or binding mechanisms involved, should be carried out in the near future.

Despite that the encapsulation system employed seems to be a promising delivery system for *A. muciniphila*, ensuring high probiotic viability levels during storage and gastrointestinal transit, future works targeting the assessment of the size and morphology of these probiotic calcium alginate capsules, should be performed. Such studies would allow to evaluate the potential application of probiotic capsules in suitable food products. Specifically, the capsules could be incorporated into cereal bars, yogurt with cereals or even bubble tea, due to the large size of these probiotic capsules (> 1mm since they are easily visualized in their individualized form by the human eye). It would be highly interesting to conduct subsequent analyses of the bioactivities exhibited by these value-added foods. Such investigations would help shed light on whether *A. muciniphila* can deliver specific benefits to consumers when consumed as part of these products.

Another extension of this work would be related with evaluation of physicochemical parameters and biological activities throughout storage at least over the 21 days. Such monitoring would provide insights into whether *A. muciniphila* is exerting any metabolic actions within the food matrix. These investigations are essential to understand the functionality and viability of *A. muciniphila* in the specific food product and its potential effects on the consumer. Furthermore, additional studies aiming the determination of nutritional composition and the monitoring of specific nutrient profiles such as amino

acids or fatty acids, as well as consumer acceptance by performing a sensorial analysis should be conducted.

6. References

Almeida, D., Machado, D., Andrade, J. C., Mendo, S., Gomes, A. M., & Freitas, A. C. (2020). Evolving trends in next-generation probiotics: a 5W1H perspective. In *Critical Reviews in Food Science and Nutrition* (Vol. 60, Issue 11, pp. 1783–1796). Taylor and Francis Inc. https://doi.org/10.1080/10408398.2019.1599812

Almeida, D., Machado, D., Sousa, S., Seabra, C. L., Barbosa, J. C., Andrade, J. C., Gomes, A. M., & Freitas, A. C. (2022). Effect of emulsification/internal gelation-based microencapsulation on the viability of *Akkermansia muciniphila* upon prolonged storage and simulated gastrointestinal passage. *Food Hydrocolloids for Health*, *2*, 100084. https://doi.org/10.1016/j.fhfh.2022.100084

Amine, K. M., Champagne, C. P., Salmieri, S., Britten, M., St-Gelais, D., Fustier, P., & Lacroix, M. (2014). Effect of palmitoylated alginate microencapsulation on viability of *Bifidobacterium longum* during freeze-drying. *LWT - Food Science and Technology*, *56*(1), 111–117. https://doi.org/10.1016/j.lwt.2013.11.003

Andrade, J. C., Almeida, D., Domingos, M., Seabra, C. L., Machado, D., Freitas, A. C., & Gomes, A. M. (2020). Commensal obligate anaerobic bacteria and health: Production, storage, and delivery strategies. *Frontiers in Bioengineering and Biotechnology*, *8*(June), 1–23. https://doi.org/10.3389/fbioe.2020.00550

Arfaoui, L. (2021). Dietary Plant Polyphenols: Effects of Food Processing on Their Content and Bioavailability. *Molecules*, *26*(10), 2959. https://doi.org/10.3390/molecules26102959

Aune, D., Norat, T., Romundstad, P., & Vatten, L. J. (2013). Dairy products and the risk of type 2 diabetes: a systematic review and dose-response meta-analysis of cohort studies. *The American Journal of Clinical Nutrition*, *98*(4), 1066–1083. https://doi.org/10.3945/ajcn.113.059030

Babot, J. D., Argañaraz-Martínez, E., Saavedra, L., Apella, M. C., & Perez Chaia, A. (2014). Selection of indigenous lactic acid bacteria to reinforce the intestinal microbiota of newly hatched chicken – relevance of in vitro and ex vivo methods for strains characterization. *Research in Veterinary Science*, *97*(1), 8–17. https://doi.org/10.1016/j.rvsc.2014.06.001

Barbosa, J., Almeida, D., Machado, D., Sousa, S., Freitas, A., Andrade, J., & Gomes, A. (2022). Spray-Drying Encapsulation of the Live Biotherapeutic Candidate *Akkermansia muciniphila* DSM 22959 to Survive Aerobic Storage. *Pharmaceuticals*, *15*(5), 628. https://doi.org/10.3390/ph15050628

Barbosa, J. C., Machado, D., Almeida, D., Andrade, J. C., Brandelli, A., Gomes, A. M., & Freitas, A. C. (2022). Next-generation probiotics. In *Probiotics* (pp. 483–502). Elsevier. https://doi.org/10.1016/B978-0-323-85170-1.00012-9

Begley, M., Gahan, C. G. M., & Hill, C. (2005). The interaction between bacteria and bile. *FEMS Microbiology Reviews*, *29*(4), 625–651. https://doi.org/10.1016/j.femsre.2004.09.003

Bhat, M. I., Sowmya, K., Kapila, S., & Kapila, R. (2020). Potential Probiotic *Lactobacillus rhamnosus* (MTCC-5897) Inhibits *Escherichia coli* Impaired Intestinal Barrier Function by Modulating the Host Tight Junction Gene Response. *Probiotics and Antimicrobial Proteins*, *12*(3), 1149–1160. https://doi.org/10.1007/s12602-019-09608-8

Binda, S., Hill, C., Johansen, E., Obis, D., Pot, B., Sanders, M. E., Tremblay, A., & Ouwehand, A. C. (2020). Criteria to Qualify Microorganisms as "Probiotic" in Foods and Dietary Supplements. Front. Microbiol. 11:1662. doi: 10.3389/fmicb.2020.01662

Bintsis, T., & Papademas, P. (2023). Sustainable Approaches in Whey Cheese Production: A Review. *Dairy*, *4*(2), 249–270. https://doi.org/10.3390/dairy4020018

Bodart, M., de Peñaranda, R., Deneyer, A., & Flamant, G. (2008). Photometry and colorimetry characterisation of materials in daylighting evaluation tools. *Building and Environment*, *43*(12), 2046–2058. https://doi.org/10.1016/j.buildenv.2007.12.006

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., Carrière, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., … Recio, I. (2019). Infogest static in vitro simulation of gastrointestinal food digestion. *Nature Protocols*, *14*(4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1

Byeon, H. R., Jang, S. Y., Lee, Y., Kim, D., Hong, M. G., Lee, D., Shin, J. H., & Seo, J. G. (2022). New Strains of *Akkermansia muciniphila* and

Faecalibacterium prausnitzii are Effective for Improving the Muscle Strength of Mice with Immobilization-Induced Muscular Atrophy. *Journal of Medicinal Food*, *25*(6), 565–575. https://doi.org/10.1089/jmf.2021.K.0148

Chandramouli, V., Kailasapathy, K., Peiris, P., & Jones, M. (2004). An improved method of microencapsulation and its evaluation to protect *Lactobacillus* spp. in simulated gastric conditions. *Journal of Microbiological Methods*, *56*(1), 27–35. https://doi.org/10.1016/j.mimet.2003.09.002

Chang, Y., Yang, Y., Xu, N., Mu, H., Zhang, H., & Duan, J. (2020). Improved viability of *Akkermansia muciniphila* by encapsulation in spray dried succinate-grafted alginate doped with epigallocatechin-3-gallate. *International Journal of Biological Macromolecules*, *159*, 373–382. https://doi.org/10.1016/j.ijbiomac.2020.05.055

Chen, Y., Zhou, J., & Wang, L. (2021). Role and Mechanism of Gut Microbiota in Human Disease. *Frontiers in Cellular and Infection Microbiology*, *11*. https://doi.org/10.3389/fcimb.2021.625913

Collado, M. C., Meriluoto, J., & Salminen, S. (2008). Adhesion and aggregation properties of probiotic and pathogen strains. *European Food Research and Technology*, *226*(5), 1065–1073. https://doi.org/10.1007/s00217-007-0632-x

Cordaillat-Simmons, M., Rouanet, A., & Pot, B. (2020). Live biotherapeutic products: the importance of a defined regulatory framework. *Experimental & Molecular Medicine*, *52*(9), 1397–1406. https://doi.org/10.1038/s12276-020-0437-6

Coscueta, E. R., Pellegrini Malpiedi, L., & Nerli, B. B. (2018). Micellar systems of aliphatic alcohol ethoxylates as a sustainable alternative to extract soybean isoflavones. *Food Chemistry*, *264*, 135–141. https://doi.org/10.1016/j.foodchem.2018.05.015

Cozzolino, A., Vergalito, F., Tremonte, P., Iorizzo, M., Lombardi, S. J., Sorrentino, E., Luongo, D., Coppola, R., & Marco, R. Di. (2020). Preliminary Evaluation of the Safety and Probiotic Potential of *Akkermansia muciniphila* DSM 22959 in Comparison with *Lactobacillus rhamnosus* GG. *Microorganisms*, *8*(2), 189. https://doi.org/10.3390/microorganisms8020189

Dadonaite, B., Ritchie, H., & Roser, M. (2018). *Diarrheal diseases.* Published online at Our World in Data.org. Retrieved from: 'https://ourworldindata.org/diarrheal-diseases' [Online Resource]

D'Archivio, M., Filesi, C., Varì, R., Scazzocchio, B., & Masella, R. (2010). Bioavailability of the Polyphenols: Status and Controversies. *International Journal of Molecular Sciences*, *11*(4), 1321–1342. https://doi.org/10.3390/ijms11041321

de Morais, J. L., Garcia, E. F., Viera, V. B., Silva Pontes, E. D., de Araújo, M. G. G., de Figueirêdo, R. M. F., dos Santos Moreira, I., do Egito, A. S., dos Santos, K. M. O., Barbosa Soares, J. K., do Egypto Queiroga, R. de C. R., & de Oliveira, M. E. G. (2022). Autochthonous adjunct culture of *Limosilactobacillus mucosae* CNPC007 improved the techno-functional, physicochemical, and sensory properties of goat milk Greek-style yogurt. *Journal of Dairy Science*, *105*(3), 1889–1899. https://doi.org/10.3168/jds.2021-21110

DeGruttola, A. K., Low, D., Mizoguchi, A., & Mizoguchi, E. (2016). Current Understanding of Dysbiosis in Disease in Human and Animal Models. *Inflammatory Bowel Diseases*, *22*(5), 1137–1150. https://doi.org/10.1097/MIB.0000000000000750

Deng Si-si, Chen Jing, Liu Xue, DONG Xin-yan, Zhang Xiang-ling, & Wang Guo-qing. (2022). [Protective Effects of *Akkermansia muciniphila* and *Amuc*_1100 Protein on Rats on High-Fat Diet Combined with Streptozotocin Injection]. *Journal of Sichuan University*. Medical science edition, 53(1), 83–91. https://doi.org/10.12182/20220160304

Derrien, M., Collado, M. C., Ben-Amor, K., Salminen, S., & De Vos, W. M. (2008). The mucin degrader *Akkermansia muciniphila* is an abundant resident of the human intestinal tract. *Applied and Environmental Microbiology*, *74*(5), 1646–1648. https://doi.org/10.1128/AEM.01226-07

Derrien, M., Vaughan, E. E., Plugge, C. M., & de Vos, W. M. (2004). *Akkermansia muciniphila* gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. *International Journal of Systematic and Evolutionary Microbiology*, *54*(5), 1469–1476. https://doi.org/10.1099/ijs.0.02873-0

Di Tommaso, N., Gasbarrini, A., & Ponziani, F. R. (2021). Intestinal Barrier in Human Health and Disease. *International Journal of Environmental Research and Public Health*, *18*(23), 12836. https://doi.org/10.3390/ijerph182312836

Distrutti, E., Monaldi, L., Ricci, P., & Fiorucci, S. (2016). Gut microbiota role in irritable bowel syndrome: New therapeutic strategies. *World Journal of Gastroenterology*, *22*(7), 2219–2241. https://doi.org/10.3748/wjg.v22.i7.2219

DSMZ. (2020). *Akkermansia muciniphila DSM 22959*.

Ekmekci, H., Aslim, B., & Ozturk, S. (2009). Characterization of vaginal lactobacilli coaggregation ability with *Escherichia coli*. *Microbiology and Immunology*, *53*(2), 59–65. https://doi.org/10.1111/j.1348-0421.2009.00115.x

Faustino, M., Machado, D., Rodrigues, D., Andrade, J. C., Freitas, A. C., & Gomes, A. M. (2023). Design and Characterization of a Cheese Spread Incorporating *Osmundea pinnatifida* Extract. *Foods*, *12*(3), 611. https://doi.org/10.3390/foods12030611

Ferreira, C. L., Grześkowiak, L., Collado, M. C., & Salminen, S. (2011). In Vitro Evaluation of *Lactobacillus gasseri* Strains of Infant Origin on Adhesion and Aggregation of Specific Pathogens. *Journal of Food Protection*, *74*(9), 1482–1487. https://doi.org/10.4315/0362-028X.JFP-11-074

Food and Drug Administration. (2016). *Early Clinical Trials with Live Biotherapeutic Products: Chemistry, Manufacturing, and Control Information*.

Frakolaki, G., Giannou, V., Topakas, E., & Tzia, C. (2021). Effect of various encapsulating agents on the beads' morphology and the viability of cells during BB-12 encapsulation through extrusion. *Journal of Food Engineering*, *294*, 110423. https://doi.org/10.1016/j.jfoodeng.2020.110423

Fusco, V., Chieffi, D., Fanelli, F., Logrieco, A. F., Cho, G., Kabisch, J., Böhnlein, C., & Franz, C. M. A. P. (2020). Microbial quality and safety of milk and milk products in the 21st century. *Comprehensive Reviews in Food Science and Food Safety*, *19*(4), 2013–2049. https://doi.org/10.1111/1541-4337.12568

Gao, D., Ning, N., Wang, C., Wang, Y., Li, Q., Meng, Z., Liu, Y., & Li, Q. (2013). Dairy Products Consumption and Risk of Type 2 Diabetes: Systematic Review and Dose-Response Meta-Analysis. *PLoS ONE*, *8*(9), e73965. https://doi.org/10.1371/journal.pone.0073965

Garcia, M. M. E., Pereira, C. J. D., Freitas, A. C., Gomes, A. M. P., & Pintado, M. M. E. (2022). Development and Characterization of a Novel Sustainable Probiotic Goat Whey Cheese Containing Second Cheese Whey Powder and Stabilized with Thyme Essential Oil and Sodium Citrate. *Foods*, *11*(17), 2698. https://doi.org/10.3390/foods11172698

Garcia-Gutierrez, E., Mayer, M. J., Cotter, P. D., & Narbad, A. (2019). Gut microbiota as a source of novel antimicrobials. *Gut Microbes*, *10*(1), 1–21. https://doi.org/10.1080/19490976.2018.1455790

Geerlings, S. Y., Kostopoulos, I., de Vos, W. M., & Belzer, C. (2018). *Akkermansia muciniphila* in the Human Gastrointestinal Tract: When, Where, and How? Microorganisms, 6(3), 75. *MDPI AG*. Retrieved from http://dx.doi.org/10.3390/microorganisms6030075

Gheorghita Puscaselu, R., Lobiuc, A., Dimian, M., & Covasa, M. (2020). Alginate: From Food Industry to Biomedical Applications and Management of Metabolic Disorders. *Polymers*, *12*(10), 2417. https://doi.org/10.3390/polym12102417

Gonçalves, B., Falco, V., Moutinho-Pereira, J., Bacelar, E., Peixoto, F., & Correia, C. (2009). Effects of Elevated CO 2 on Grapevine (Vitis vinifera L.): Volatile Composition, Phenolic Content, and in Vitro Antioxidant Activity of Red Wine. *Journal of Agricultural and Food Chemistry*, *57*(1), 265–273. https://doi.org/10.1021/jf8020199

Gyawali, R., Feng, X., Chen, Y. P., Lorenzo, J. M., & Ibrahim, S. A. (2022). A review of factors influencing the quality and sensory evaluation techniques applied to Greek yogurt. *Journal of Dairy Research*, *89*(2), 213–219. https://doi.org/10.1017/S0022029922000346

Heidari, Z., Rashidi Pour Fard, N., Clark, C. C. T., & Haghighatdoost, F. (2021). Dairy products consumption and the risk of hypertension in adults: An updated systematic review and dose–response meta-analysis of prospective cohort studies. *Nutrition, Metabolism and Cardiovascular Diseases*, *31*(7), 1962–1975. https://doi.org/10.1016/j.numecd.2021.02.033

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C., & Sanders, M. E. (2014). Expert consensus document: The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology and Hepatology*, *11*(8), 506–514. https://doi.org/10.1038/nrgastro.2014.66

Hough, G., Puglieso, M. L., Sanchez, R., & da Silva, O. M. (1999). Sensory and Microbiological Shelf-Life of a Commercial Ricotta Cheese. *Journal of Dairy Science*, *82*(3), 454–459. https://doi.org/10.3168/jds.S0022-0302(99)75253-7

International Diabetes Federation. (2022). *Diabetes around the world in 2021*.

Jena, P. K., Trivedi, D., Thakore, K., Chaudhary, H., Giri, S. S., & Seshadri, S. (2013). Isolation and characterization of probiotic properties of lactobacilli isolated from rat fecal microbiota. *Microbiology and Immunology*, *57*(6), 407–416. https://doi.org/10.1111/1348-0421.12054

Kaur, H., Kaur, G., & Ali, S. A. (2022). Dairy-Based Probiotic-Fermented Functional Foods: An Update on Their Health-Promoting Properties. *Fermentation*, *8*(9), 425. https://doi.org/10.3390/fermentation8090425

Kechagia, M., Basoulis, D., Konstantopoulou, S., Dimitriadi, D., Gyftopoulou, K., Skarmoutsou, N., & Fakiri, E. M. (2013). Health benefits of probiotics: a review. *ISRN Nutrition*, *2013*, 481651. https://doi.org/10.5402/2013/481651

Khan, I. T., Nadeem, M., Imran, M., Ajmal, M., & Ali, S. (2018). Antioxidant activity, fatty acids characterization and oxidative stability of Gouda cheese fortified with mango (Mangifera indica L.) kernel fat. *Journal of Food Science and Technology*, *55*(3), 992–1002. https://doi.org/10.1007/s13197-017-3012-y

Khoddami, A., Wilkes, M., & Roberts, T. (2013). Techniques for Analysis of Plant Phenolic Compounds. *Molecules*, *18*(2), 2328–2375. https://doi.org/10.3390/molecules18022328

Koh, W. Y., Lim, X. X., Tan, T.-C., Kobun, R., & Rasti, B. (2022). Encapsulated Probiotics: Potential Techniques and Coating Materials for Non-Dairy Food Applications. *Applied Sciences*, *12*(19), 10005. https://doi.org/10.3390/app121910005

Kopacz, K., & Phadtare, S. (2022). Probiotics for the Prevention of Antibiotic-Associated Diarrhea. Healthcare, 10(8), 1450. MDPI AG. Retrieved from http://dx.doi.org/10.3390/healthcare10081450

Kos, B., Šušković, J., Vuković, S., Šimpraga, M., Frece, J., & Matošić, S. (2003). Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *Journal of Applied Microbiology*, *94*(6), 981–987. https://doi.org/10.1046/j.1365-2672.2003.01915.x

Krausova, G., Hyrslova, I., & Hynstova, I. (2019). In vitro evaluation of adhesion capacity, hydrophobicity, and auto-aggregation of newly isolated potential probiotic strains. *Fermentation*, *5*(4). https://doi.org/10.3390/fermentation5040100

Kwon, Y.-I., Apostolidis, E., & Shetty, K. (2008). In vitro studies of eggplant (Solanum melongena) phenolics as inhibitors of key enzymes relevant for type

2 diabetes and hypertension. *Bioresource Technology*, *99*(8), 2981–2988. https://doi.org/10.1016/j.biortech.2007.06.035

Lange, K., Buerger, M., Stallmach, A., & Bruns, T. (2016). Effects of Antibiotics on Gut Microbiota. In *Digestive Diseases* (Vol. 34, Issue 3, pp. 260–268). S. Karger AG. https://doi.org/10.1159/000443360

Lee, K.-Y., & Heo, T.-R. (2000). Survival of *Bifidobacterium longum* Immobilized in Calcium Alginate Beads in Simulated Gastric Juices and Bile Salt Solution. *Applied and Environmental Microbiology*, *66*(2), 869–873. https://doi.org/10.1128/AEM.66.2.869-873.2000

Lee, Y., Byeon, H. R., Jang, S. Y., Hong, M. G., Kim, D., Lee, D., Shin, J. H., Kim, Y., Kang, S. G., & Seo, J. G. (2022). Oral administration *of Faecalibacterium prausnitzii* and *Akkermansia muciniphila* strains from humans improves atopic dermatitis symptoms in DNCB induced NC/Nga mice. *Scientific Reports*, *12*(1). https://doi.org/10.1038/s41598-022-11048-4

Lilly, D. M., & Stillwell, R. H. (1965). Probiotics: Growth-promoting factors produced by microorganisms. *Science*, *147*(3659), 747–748. https://doi.org/10.1126/science.147.3659.747

Lin, D., Xiao, M., Zhao, J., Li, Z., Xing, B., Li, X., Kong, M., Li, L., Zhang, Q., Liu, Y., Chen, H., Qin, W., Wu, H., & Chen, S. (2016). An Overview of Plant Phenolic Compounds and Their Importance in Human Nutrition and Management of Type 2 Diabetes. *Molecules*, *21*(10), 1374. https://doi.org/10.3390/molecules21101374

Liu, J., Liu, H., Liu, H., Teng, Y., Qin, N., Ren, X., & Xia, X. (2023). Live and pasteurized *Akkermansia muciniphila* decrease susceptibility to *Salmonella Typhimurium* infection in mice. *Journal of Advanced Research*. https://doi.org/10.1016/j.jare.2023.03.008

Machado, D., Almeida, D., Seabra, C. L., Andrade, J. C., Gomes, A. M., & Freitas, A. C. (2020). Nanoprobiotics: When Technology Meets Gut Health. In *Functional Bionanomaterials* (pp. 389–425). Springer. https://doi.org/10.1007/978-3-030-41464-1_17

Madureira, A. R., Amorim, M., Gomes, A. M., Pintado, M. E., & Malcata, F. X. (2011). Protective effect of whey cheese matrix on probiotic strains exposed to simulated gastrointestinal conditions. *Food Research International*, *44*(1), 465–470. https://doi.org/10.1016/j.foodres.2010.09.010

Madureira, A. R., Gião, M. S., Pintado, M. E., Gomes, A. M. P., Freitas, A. C., & Malcata, F. X. (2006). Incorporation and Survival of Probiotic Bacteria in Whey Cheese Matrices. *Journal of Food Science*, *70*(3), M160–M165. https://doi.org/10.1111/j.1365-2621.2005.tb07144.x

Madureira, A. R., Pintado, M. E., Gomes, A. M. P., & Malcata, F. X. (2011). Incorporation of Probiotic Bacteria in Whey Cheese: Decreasing the Risk of Microbial Contamination. *Journal of Food Protection*, *74*(7), 1194–1199. https://doi.org/10.4315/0362-028X.JFP-10-217

Madureira, A. R., Soares, J. C., Pintado, M. E., Gomes, A. M. P., Freitas, A. C., & Malcata, F. X. (2008). Sweet whey cheese matrices inoculated with the probiotic strain *Lactobacillus paracasei* LAFTI ® L26. *Dairy Science and Technology*, *88*(6), 649–665. https://doi.org/10.1051/dst:2008026

Madureira, A. R., Soares, J. C., Pintado, M. E., Gomes, A. M. P., Freitas, A. C., & Xavier Malcata, F. (2015). Effect of the incorporation of salted additives on probiotic whey cheeses. *Food Bioscience*, *10*, 8–17. https://doi.org/10.1016/j.fbio.2015.01.004

Marcial-Coba, M. S., Cieplak, T., Cahú, T. B., Blennow, A., Knøchel, S., & Nielsen, D. S. (2018). Viability of microencapsulated *Akkermansia muciniphila* and *Lactobacillus plantarum* during freeze-drying, storage and *in vitro* simulated upper gastrointestinal tract passage. *Food & Function*, *9*(11), 5868–5879. https://doi.org/10.1039/C8FO01331D

Marcial-Coba, M. S., Saaby, L., Knøchel, S., & Nielsen, D. S. (2019). Dark chocolate as a stable carrier of microencapsulated *Akkermansia muciniphila* and *Lactobacillus casei*. *FEMS Microbiology Letters*, *366*(2). https://doi.org/10.1093/femsle/fny290

Martin, M. J., Lara-Villoslada, F., Ruiz, M. A., & Morales, M. E. (2013). Effect of unmodified starch on viability of alginate-encapsulated *Lactobacillus fermentum* CECT5716. *LWT - Food Science and Technology*, *53*(2), 480–486. https://doi.org/10.1016/j.lwt.2013.03.019

Martín, R., & Langella, P. (2019). Emerging Health Concepts in the Probiotics Field: Streamlining the Definitions. Front. Microbiol. 10:1047. doi: 10.3389/fmicb.2019.01047

Metchnikoff, E. (1908). The Prolongation of Life. *Nature*, *77*(1996), 289–290. https://doi.org/10.1038/077289b0

Moineau-Jean, A., Champagne, C. P., Roy, D., Raymond, Y., & LaPointe, G. (2019). Effect of Greek-style yoghurt manufacturing processes on starter and probiotic bacteria populations during storage. *International Dairy Journal*, *93*, 35–44. https://doi.org/10.1016/j.idairyj.2019.02.003

Mulhall, H., DiChiara, J. M., Huck, O., & Amar, S. (2022). Pasteurized *Akkermansia muciniphila* reduces periodontal and systemic inflammation induced by *Porphyromonas gingivalis* in lean and obese mice. *Journal of Clinical Periodontology*, *49*(7), 717–729. https://doi.org/10.1111/jcpe.13629

Nardini, M. (2022). Phenolic Compounds in Food: Characterization and Health Benefits. *Molecules*, *27*(3), 783. https://doi.org/10.3390/molecules27030783

Neef, A., & Sanz, Y. (2013). Future for probiotic science in functional food and dietary supplement development. Current opinion in clinical nutrition and metabolic care, 16(6), 679–687. https://doi.org/10.1097/MCO.0b013e328365c258

O'Connell, J. E., & Fox, P. F. (2001). Significance and applications of phenolic compounds in the production and quality of milk and dairy products: a review. *International Dairy Journal*, *11*(3), 103–120. https://doi.org/10.1016/S0958-6946(01)00033-4

O'Toole, P. W., Marchesi, J. R., & Hill, C. (2017). Next-generation probiotics: the spectrum from probiotics to live biotherapeutics. Nature microbiology, 2, 17057. https://doi.org/10.1038/nmicrobiol.2017.57

Pellegrino, A., Coppola, G., Santopaolo, F., Gasbarrini, A., & Ponziani, F. R. (2023). Role of Akkermansia in Human Diseases: From Causation to Therapeutic Properties. *Nutrients*, *15*(8), 1815. https://doi.org/10.3390/nu15081815

Petschacher, B., & Nidetzky, B. (2016). Biotechnological production of fucosylated human milk oligosaccharides: Prokaryotic fucosyl transferases and their use in biocatalytic cascades or whole cell conversion systems. *Journal of Biotechnology*, *235*, 61–83. https://doi.org/10.1016/j.jbiotec.2016.03.052

Petti, S., & Scully, C. (2009). Polyphenols, oral health and disease: A review. *Journal of Dentistry*, *37*(6), 413–423. https://doi.org/10.1016/j.jdent.2009.02.003

Pintado, M. E., Macedo, A. C., & Malcata, F. X. (2001). Review: Technology, Chemistry and Microbiology of Whey Cheeses. *Food Science and Technology International*, *7*(2), 105–116. https://doi.org/10.1177/108201320100700202

Pinto, S. S., Cavalcante, B. D., Verruck, S., Alves, L. F., Prudêncio, E. S., & Amboni, R. D. (2017). Effect of the incorporation of *Bifidobacterium* BB-12 microencapsulated with sweet whey and inulin on the properties of Greek-style yogurt. *Journal of Food Science and Technology*, *54*(9), 2804–2813. https://doi.org/10.1007/s13197-017-2717-2

Pitino, I., Randazzo, C. L., Cross, K. L., Parker, M. L., Bisignano, C., Wickham, M. S. J., Mandalari, G., & Caggia, C. (2012). Survival of *Lactobacillus rhamnosus* strains inoculated in cheese matrix during simulated human digestion. *Food Microbiology*, *31*(1), 57–63. https://doi.org/10.1016/j.fm.2012.02.013

Plaza-Diaz, J., Ruiz-Ojeda, F. J., Gil-Campos, M., & Gil, A. (2019). Mechanisms of Action of Probiotics. *Advances in Nutrition*, *10*, S49–S66. https://doi.org/10.1093/advances/nmy063

Raftar, S. K. A., Ashrafian, F., Abdollahiyan, S., Yadegar, A., Moradi, H. R., Masoumi, M., Vaziri, F., Moshiri, A., Siadat, S. D., & Zali, M. R. (2022). The anti-inflammatory effects of *Akkermansia muciniphila* and its derivates in HFD/CCL4-induced murine model of liver injury. *Scientific Reports*, *12*(1). https://doi.org/10.1038/s41598-022-06414-1

Ramirez, J., Guarner, F., Bustos Fernandez, L., Maruy, A., Sdepanian, V. L., & Cohen, H. (2020). Antibiotics as Major Disruptors of Gut Microbiota. In *Frontiers in Cellular and Infection Microbiology* (Vol. 10). Frontiers Media S.A. https://doi.org/10.3389/fcimb.2020.572912

Ribeiro, T. B., Bonifácio-Lopes, T., Morais, P., Miranda, A., Nunes, J., Vicente, A. A., & Pintado, M. (2021). Incorporation of olive pomace ingredients into yoghurts as a source of fibre and hydroxytyrosol: Antioxidant activity and stability throughout gastrointestinal digestion. *Journal of Food Engineering*, *297*, 110476. https://doi.org/10.1016/j.jfoodeng.2021.110476

Rolain, J. M. (2013). Food and human gut as reservoirs of transferable antibiotic resistance encoding genes. In *Frontiers in Microbiology* (Vol. 4, Issue JUN). Frontiers Research Foundation. https://doi.org/10.3389/fmicb.2013.00173

Rolim, F. R. L., Freitas Neto, O. C., Oliveira, M. E. G., Oliveira, C. J. B., & Queiroga, R. C. R. E. (2020). Cheeses as food matrixes for probiotics: In vitro and in vivo tests. *Trends in Food Science & Technology*, *100*, 138–154. https://doi.org/10.1016/j.tifs.2020.04.008

Rowland, I., Gibson, G., Heinken, A., Scott, K., Swann, J., Thiele, I., & Tuohy, K. (2018). Gut microbiota functions: metabolism of nutrients and other food components. *European Journal of Nutrition*, *57*(1), 1–24. https://doi.org/10.1007/s00394-017-1445-8

Sánchez, B., Delgado, S., Blanco-Míguez, A., Lourenço, A., Gueimonde, M., & Margolles, A. (2017). robiotics, gut microbiota, and their influence on host health and disease. Molecular nutrition & food research, 61(1), 10.1002/mnfr.201600240. https://doi.org/10.1002/mnfr.201600240

Schneeberger, M., Everard, A., Gómez-Valadés, A. G., Matamoros, S., Ramírez, S., Delzenne, N. M., Gomis, R., Claret, M., & Cani, P. D. (2015). *Akkermansia muciniphila* inversely correlates with the onset of inflammation, altered adipose tissue metabolism and metabolic disorders during obesity in mice. *Scientific Reports*, *5*. https://doi.org/10.1038/srep16643

Sentandreu, M., & Toldra, F. (2006). A rapid, simple and sensitive fluorescence method for the assay of angiotensin-I converting enzyme. *Food Chemistry*, *97*(3), 546–554. https://doi.org/10.1016/j.foodchem.2005.06.006

Sheehan, D., Moran, C., & Shanahan, F. (2015). The microbiota in inflammatory bowel disease. *Journal of Gastroenterology*, *50*(5), 495–507. https://doi.org/10.1007/s00535-015-1064-1

Shui, G., & Leong, L. P. (2002). Separation and determination of organic acids and phenolic compounds in fruit juices and drinks by high-performance liquid chromatography. *Journal of Chromatography A*, *977*(1), 89–96. https://doi.org/10.1016/S0021-9673(02)01345-6

Singh, S., Gupta, R., Chawla, S., Gauba, P., Singh, M., Tiwari, R. K., Upadhyay, S., Sharma, S., Chanda, S., & Gaur, S. (2022). Natural sources and encapsulating materials for probiotics delivery systems: Recent applications and challenges in functional food development. *Frontiers in Nutrition*, *9*. https://doi.org/10.3389/fnut.2022.971784

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *American Journal of Enology and Viticulture*, *16*(3), 144–158. https://doi.org/10.5344/ajev.1965.16.3.144

Song, F.-L., Gan, R.-Y., Zhang, Y., Xiao, Q., Kuang, L., & Li, H.-B. (2010). Total Phenolic Contents and Antioxidant Capacities of Selected Chinese Medicinal

Plants. *International Journal of Molecular Sciences*, *11*(6), 2362–2372. https://doi.org/10.3390/ijms11062362

Soni, A., Samuelsson, L. M., Loveday, S. M., & Gupta, T. B. (2021). Applications of novel processing technologies to enhance the safety and bioactivity of milk. *Comprehensive Reviews in Food Science and Food Safety*, *20*(5), 4652–4677. https://doi.org/10.1111/1541-4337.12819

Sousa, S., Gomes, A. M., Pintado, M. M., Malcata, F. X., Silva, J. P., Sousa, J. M., Costa, P., Amaral, M. H., Rodrigues, D., Rocha-santos, T. A. P., & Freitas, A. C. (2012). Encapsulation of probiotic strains in plain or cysteine-supplemented alginate improves viability at storage below freezing temperatures. *Engineering in Life Sciences*, *12*(4), 457–465. https://doi.org/10.1002/elsc.201200007

Srinivasan, B., Kolli, A. R., Esch, M. B., Abaci, H. E., Shuler, M. L., & Hickman, J. J. (2015). TEER Measurement Techniques for In Vitro Barrier Model Systems. *SLAS Technology*, *20*(2), 107–126. https://doi.org/10.1177/2211068214561025

Stanton, C., Gardiner, G., Lynch, P. B., Collins, J. K., Fitzgerald, G., & Ross, R. P. (1998). Probiotic Cheese. *International Dairy Journal*, *8*(5–6), 491–496. https://doi.org/10.1016/S0958-6946(98)00080-6

Terpou, A., Papadaki, A., Lappa, I., Kachrimanidou, V., Bosnea, L., & Kopsahelis, N. (2019). Probiotics in Food Systems: Significance and Emerging Strategies Towards Improved Viability and Delivery of Enhanced Beneficial Value. *Nutrients*, *11*(7), 1591. https://doi.org/10.3390/nu11071591

Thursby, E., & Juge, N. (2017). Introduction to the human gut microbiota. *Biochemical Journal*, *474*(11), 1823–1836. https://doi.org/10.1042/BCJ20160510

Troeger, C., Blacker, B. F., Khalil, I. A., Rao, P. C., Cao, S., Zimsen, S. R., Albertson, S. B., Stanaway, J. D., Deshpande, A., Abebe, Z., Alvis-Guzman, N., Amare, A. T., Asgedom, S. W., Anteneh, Z. A., Antonio, C. A. T., Aremu, O., Asfaw, E. T., Atey, T. M., Atique, S., … Reiner, R. C. (2018). Estimates of the global, regional, and national morbidity, mortality, and aetiologies of diarrhoea in 195 countries: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet Infectious Diseases*, *18*(11), 1211–1228. https://doi.org/10.1016/S1473-3099(18)30362-1

Tuo, Y., Yu, H., Ai, L., Wu, Z., Guo, B., & Chen, W. (2013). Aggregation and adhesion properties of 22 Lactobacillus strains. *Journal of Dairy Science*, *96*(7), 4252–4257. https://doi.org/10.3168/jds.2013-6547

van der Ark, K. C. H., Nugroho, A. D. W., Berton-Carabin, C., Wang, C., Belzer, C., de Vos, W. M., & Schroen, K. (2017). Encapsulation of the therapeutic microbe *Akkermansia muciniphila* in a double emulsion enhances survival in simulated gastric conditions. *Food Research International*, *102*, 372–379. https://doi.org/10.1016/j.foodres.2017.09.004

van Zyl, W. F., Deane, S. M., & Dicks, L. M. T. (2020). Molecular insights into probiotic mechanisms of action employed against intestinal pathogenic bacteria. *Gut Microbes*, *12*(1), 1831339. https://doi.org/10.1080/19490976.2020.1831339

Verdu, E. F., Galipeau, H. J., & Jabri, B. (2015). Novel players in coeliac disease pathogenesis: Role of the gut microbiota. *Nature Reviews Gastroenterology and Hepatology*, *12*(9), 497–506. https://doi.org/10.1038/nrgastro.2015.90

Voss, G. B., Machado, D., Barbosa, J. C., Campos, D. A., Gomes, A. M., & Pintado, M. (2022). Interplay between probiotics and prebiotics for human nutrition and health. In *Probiotics for Human Nutrition in Health and Disease* (pp. 231–254). Elsevier. https://doi.org/10.1016/B978-0-323-89908-6.00027-3

Wan, Z., Zheng, J., Zhu, Z., Sang, L., Zhu, J., Luo, S., Zhao, Y., Wang, R., Zhang, Y., Hao, K., Chen, L., Du, J., Kan, J., & He, H. (2022). Intermediate role of gut microbiota in vitamin B nutrition and its influences on human health. *Frontiers in Nutrition*, *9*. https://doi.org/10.3389/fnut.2022.1031502

West, C. E., Renz, H., Jenmalm, M. C., Kozyrskyj, A. L., Allen, K. J., Vuillermin, P., Prescott, S. L., MacKay, C., Salminen, S., Wong, G., Sinn, J., Stokholm, J., Bisgaard, H., Pawankar, R., Noakes, P., Kesper, D., & Tulic, M. (2015). The gut microbiota and inflammatory noncommunicable diseases: Associations and potentials for gut microbiota therapies. *Journal of Allergy and Clinical Immunology*, *135*(1), 3–13. https://doi.org/10.1016/j.jaci.2014.11.012

World and Health Organization. (2023). *Diabetes*. https://www.who.int/news-room/fact-sheets/detail/diabetes

World and Health Organization. (2023). *Hypertension*. https://www.who.int/news-room/fact-sheets/detail/hypertension

World Health Organization. (2021). *Antimicrobial resistance*. https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance

World Health Organization, & Food and Agriculture Organization of the United Nations. (2006). *Probiotics in food Health and nutritional properties and guidelines for evaluation FAO FOOD AND NUTRITION PAPER*.

Wu, Z., Xu, Q., Gu, S., Chen, Y., Lv, L., Zheng, B., Wang, Q., Wang, K., Wang, S., Xia, J., Yang, L., Bian, X., Jiang, X., Zheng, L., & Li, L. (2022). Akkermansia muciniphila Ameliorates Clostridioides difficile Infection in Mice by Modulating the Intestinal Microbiome and Metabolites. *Frontiers in Microbiology*, *13*. https://doi.org/10.3389/fmicb.2022.841920

Xie, A., Zhao, S., Liu, Z., Yue, X., Shao, J., Li, M., & Li, Z. (2023). Polysaccharides, proteins, and their complex as microencapsulation carriers for delivery of probiotics: A review on carrier types and encapsulation techniques. *International Journal of Biological Macromolecules*, *242*, 124784. https://doi.org/10.1016/j.ijbiomac.2023.124784

Xu, H., Jeong, H. S., Lee, H. Y., & Ahn, J. (2009). Assessment of cell surface properties and adhesion potential of selected probiotic strains. *Letters in Applied Microbiology*, *49*(4), 434–442. https://doi.org/10.1111/j.1472-765X.2009.02684.x

Yang, S.-Y., & Yoon, K.-S. (2022). Effect of Probiotic Lactic Acid Bacteria (LAB) on the Quality and Safety of Greek Yogurt. *Foods*, *11*(23), 3799. https://doi.org/10.3390/foods11233799

Zhang, J., Ni, Y., Qian, L., Fang, Q., Zheng, T., Zhang, M., Gao, Q., Zhang, Y., Ni, J., Hou, X., Bao, Y., Kovatcheva-Datchary, P., Xu, A., Li, H., Panagiotou, G., & Jia, W. (2021). Decreased Abundance of *Akkermansia muciniphila* Leads to the Impairment of Insulin Secretion and Glucose Homeostasis in Lean Type 2 Diabetes. *Advanced Science*, *8*(16), 2100536. https://doi.org/10.1002/advs.202100536

Zhang, T., Li, Q., Cheng, L., Buch, H., & Zhang, F. (2019). *Akkermansia muciniphila* is a promising probiotic. Microbial biotechnology, 12(6), 1109–1125. https://doi.org/10.1111/1751-7915.13410

Zheng, J., Wittouck, S., Salvetti, E., Franz, C. M. A. P., Harris, H. M. B., Mattarelli, P., O'Toole, P. W., Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G. E., Gänzle, M. G., & Lebeer, S. (2020). A taxonomic note on the

genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. *International Journal of Systematic and Evolutionary Microbiology*, *70*(4), 2782–2858. https://doi.org/10.1099/ijsem.0.004107

Zielińska, D., Długosz, E., & Zawistowska-Deniziak, A. (2019). Functional Properties of Food Origin Lactobacillus in the Gastrointestinal Ecosystem—In Vitro Study. *Probiotics and Antimicrobial Proteins*, *11*(3), 820–829. https://doi.org/10.1007/s12602-018-9458-z