DEVELOPMENT OF PROBIOTIC BAR WITH MICROENCAPSULATED PROBIOTICS

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ABSTRACT

DEVELOPMENT OF PROBIOTIC BAR WITH MICROENCAPSULATED PROBIOTICS

The development of a probiotic bar incorporating microencapsulated probiotics, specifically *Saccharomyces boulardii* CNCM-I745 and *Alkalihalobacillus clausii* (N/R, O/C, SIN, T), was investigated to enhance the stability and viability of these beneficial microorganisms. Microencapsulation was achieved using a lupin protein isolate-xanthan gum-trehalose complex, which provided a protective matrix for the probiotics. The encapsulated probiotics were added to a bar formulation consisting of gluten-free oats, dates, peanut butter, dried figs, walnuts, flax seeds, cinnamon, and salt. Physicochemical analyses, including moisture content, water activity, texture, and color measurements, were performed to evaluate the quality of the bars. Additionally, a 90-day storage study at 4°C was conducted to monitor the viability of the probiotics. Results demonstrated that microencapsulation significantly enhanced the stability and survival rate of the probiotics, with viable counts remaining above 10^6 CFU/g throughout storage, meeting the Turkish Food Codex standards. Specifically, microencapsulated probiotics showed a log reduction of less than 1 log CFU/g, whereas free probiotics exhibited a reduction of over 3 log CFU/g during the 90-day storage. The study also examined the *in vitro* gastrointestinal survival of the probiotics, indicating that microencapsulated probiotics had superior resistance to simulated gastric and intestinal conditions. The microencapsulation process improved the retention of probiotic viability by protecting them from harsh environmental factors encountered during storage and digestion. This thesis study highlights the potential of microencapsulation techniques in developing functional foods with enhanced probiotic stability. The findings provide valuable insights for the food industry in creating innovative non-dairy probiotic products that deliver health benefits.

ÖZET

MİKROENKAPSÜLE PROBİYOTİKLER İLE PROBİYOTİK BAR GELİŞTİRİLMESİ

Bu çalışma, *Saccharomyces boulardii* CNCM-I745 ve *Alkalihalobacillus clausii* (N/R, O/C, SIN, T) gibi probiyotikleri mikroenkapsüle ederek, bu yararlı mikroorganizmaların stabilitesini ve canlılığını artırmayı amaçlayan bir probiyotik barın geliştirilmesini incelemektedir. Mikroenkapsülasyon, probiyotikleri koruyucu bir matris sağlayan lupin protein izolatı-ksantan gam-trehaloz kompleksi kullanılarak gerçekleştirilmiştir. Mikroenkapsüle edilmiş probiyotikler, glutensiz yulaf, hurma, fıstık ezmesi, kuru incir, ceviz, keten tohumu, tarçın ve tuz içeren bir bar formülasyonuna eklenmiştir. Nem içeriği, su aktivitesi, doku ve renk ölçümleri gibi fizikokimyasal analizler, barların kalitesini değerlendirmek için yapılmıştır. Ayrıca, probiyotiklerin canlılığını izlemek amacıyla 4°C'de 90 günlük bir depolama çalışması yürütülmüştür. Sonuçlar, mikroenkapsülasyonun probiyotiklerin stabilitesini ve hayatta kalma oranını önemli ölçüde artırdığını, canlı sayılarının depolama süresi boyunca 10^6 CFU/g üzerinde kaldığını ve Türk Gıda Kodeksi standartlarını karşıladığını göstermiştir. Özellikle, mikroenkapsüle edilmiş probiyotikler 90 günlük depolama süresince 1 log CFU/g'den az bir azalma gösterirken, serbest probiyotikler 3 log CFU/g'den fazla bir azalma göstermiştir. Çalışma ayrıca, probiyotiklerin in vitro gastrointestinal hayatta kalma oranlarını incelemiş ve mikroenkapsüle edilmiş probiyotiklerin simüle edilmiş mide ve bağırsak koşullarına karşı üstün direnç gösterdiğini ortaya koymuştur. Mikroenkapsülasyon süreci, depolama ve sindirim sırasında karşılaşılan zorlu çevresel faktörlerden koruyarak probiyotiklerin canlılığının korunmasını iyileştirmiştir. Bu araştırma, mikroenkapsülasyon tekniklerinin fonksiyonel gıdaların geliştirilmesindeki potansiyelini vurgulamakta ve yenilikçi, süt içermeyen probiyotik ürünler yaratma konusunda gıda endüstrisine değerli bilgiler sağlamaktadır.

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ACE	Angiotensin Converting Enzyme
ANOVA	Analysis of Variance
B. clausii	*Bacillus clausii*
CFU	Colony-forming unit
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization
GIT	Gastrointestinal tract
LbL	layer-by-layer
LPI	Lupin Protein Isolate
O/W	oil-in-water
PB C	Control bar samples (devoid of microorganisms).
PB F BC	Probiotic bar including lyophilized free *B. clausii*
PB F SB	Probiotic bar including lyophilized free *S. boulardii*
PB ME	Probiotic bar including microencapsulated *S. boulardii* & *B.clausii*
PB ME BC	Probiotic bar including microencapsulated and lyophilized *B. clausii*
PB ME SB	Probiotic bar including microencapsulated and lyophilized *S. boulardii*
S. boulardii	*Saccharomyces boulardii*
TPA	Texture Profile Analysis
W/O	water-in-oil
W/O/W	water-in-oil-in-water
WHO	World Health Organization
XG	Xanthan Gum

CHAPTER 1

INTRODUCTION

1.1. Probiotic Microorganisms

Probiotics, a field experiencing swift growth in microbiology and human health, has garnered significant attention and importance in recent years. (Gill et al., 2022). These biologically active substances, consisting of living microorganisms, provide a promising approach to modifying the human microbiota and supporting overall health (Craig & Brothers, 2021). Probiotic microorganisms, typically comprising strains of *Lactobacillus* and *Bifidobacterium*, are known for their rod-shaped, gram-positive, non-sporulating, and catalase-negative properties (Er et al., 2019). These microorganisms produce lactic acid and have been studied for their potential in promoting health, including improving gastrointestinal function, enhancing immune responses, and reducing the risk of various disorders (Brutscher et al., 2022). This expanding field of study emphasises the complex relationship between the gut microbiome and the host's physiology.

Probiotic microorganisms' function through various mechanisms, such as the competitive exclusion of pathogenic species, modulation of immune responses, and production of bioactive metabolites (McFarland et al., 2018). Moreover, they interact with the host's epithelial cells and mucosal immune system, thereby impacting the composition and stability of the gut microbiota (Hirschberg et al., 2019). To maintain gut homeostasis, it is essential to comprehend the complex connections between probiotics and the host's well-being, which can pave the way for disease prevention and management (Grumet et al., 2020). The intake of probiotics that can induce such impacts is conventionally recognised to be a minimum of $10^8 – 10^9$ CFU/g on a daily basis (Τέρπου et al., 2019). Probiotic microorganisms encounter substantial challenges that endanger their survival, such as the acidic nature of the digestive system, exposure to oxygen,

digestive enzymes, and bile salts (Han et al., 2016). To bolster their resilience against these negative conditions, current research efforts strive to boost the durability of probiotic microorganisms, whether in unconstrained form or within merchandise (Han et al., 2016). Furthermore, the creation of bespoke probiotic formulas and advanced delivery techniques has widened the scope of probiotic microorganisms, increasing their potential applicability beyond digestive health to areas like psychological welfare and skin disorders (Grumet et al., 2020; D'Egidio et al., 2022; Lemieux-Labonté et al., 2016; Jeżewska-Frąckowiak et al., 2021).

Nowadays, probiotics are incorporated into both dairy and non-dairy products for commercial use, including yogurt, probiotic milk, oat-based items, fruit and vegetable juices, and ice cream (Camelo-Silva et al., 2022; Craig & Brothers, 2021; Calumba et al., 2021; Nguyen et al., 2022). The employment of probiotics has fascinated scientific researchers as well as captured consumer interest, establishing them as a significant influence in the field of global functional food and microbiology. Consequently, probiotics, complemented by their associated microorganisms, create a multifaceted, dynamic domain with potential for enhancing human health and overall well-being.

1.2. *Saccharomyces cerevisiae* var. *boulardii*

Saccharomyces cerevisiae var. *boulardii* plays an essential role in microbial therapeutics (Adeleke & Jelilat, 2022). This unique and promising probiotic yeast was first discovered in the 1920s by French scientist Henri Boulard, during a cholera outbreak in the India-China region (Łukaszewicz, 2012). Boulard noticed that individuals who consumed tropical fruits like lychee and mango, or teas brewed from these fruits, did not experience cholera symptoms thus he isolated *S. boulardii* (Łukaszewicz, 2012). This strain of yeast, a variation of the commonly used *Saccharomyces cerevisiae* baker's yeast, has been subject to extensive study and is acknowledged for its potential therapeutic benefits in the realm of gastrointestinal health (Borșa et al., 2022). *S. boulardii* is a subspecies from the *Saccharomyces cerevisiae* species and holds a distinguished position as a probiotic, owing to its singular attributes (Borșa et al., 2022). The key contrasts between *S. boulardii* and *S. cerevisiae* are that *S. boulardii* thrives at 37 °C, similar to

human body temperature, can endure low pH values, cannot use galactose and is unable to form ascospores (Fietto et al., 2004). It is worth noting that, despite being originally identified as a distinct yeast to *S. cerevisiae*, genetic studies have demonstrated that *S. boulardii* and *S. cerevisiae* are identical, but differ significantly in their metabolism and probiotic characteristics (McFarland, 1996). Current taxonomic studies reveal that *S. boulardii* cannot be classified as a distinct species and must be acknowledged as *Saccharomyces cerevisiae* var. *boulardii* (Smith et al., 2016). Its exceptional resistance to the acidic conditions of the stomach facilitates its survival and proliferation in the gastrointestinal tract (Ghorbani-Choboghlo et al., 2019). This endurance is of utmost significance in the context of probiotic supplementation since numerous other probiotic strains face difficulties in enduring the rough circumstances of the digestive system (Ghorbani-Choboghlo et al., 2019). Moreover, it attaches to the intestinal lining and may aid in regulating the gut microbiota and producing advantageous impacts.The primary mode of action of *S. boulardii* lies in its ability to modulate the gut microbiota (Yu et al., 2017). It acts as a biotherapeutic agent, helping to maintain a balanced intestinal microbial ecosystem (Yu et al., 2017). This strain promotes the growth of beneficial bacteria and inhibits the proliferation of harmful pathogens, thus promoting gut health and reducing the risk of infections (Tao et al., 2021). It also enhances the gut's barrier function, preventing the translocation of harmful substances into the bloodstream (Carneiro et al., 2022). *S. boulardii*'s clinical applications extend beyond its gut health benefits.

This probiotic strain has demonstrated efficacy in treating various gastrointestinal conditions, including antibiotic-associated diarrhea, *Clostridium difficile* infection, and inflammatory bowel diseases (Boiocchi et al., 2013). For example, it has been shown that the 63-kDa protein phosphotase secreted by *S. boulardii* can dephosphorylate and partially inactivate *Escherichia coli* endotoxins. In addition, the 54-kDa serine protease secreted by *S. boulardii* digests toxins A and B of *Clostridium difficile* (Castagliuolo et al., 1996). Moreover, it has been explored for its potential in preventing traveler's diarrhea and mitigating the side effects of *Helicobacter pylori* eradication therapy (Behçet & Kaya, 2020; Qu et al., 2022). Its mechanisms of action are multifaceted, involving the production of antimicrobial peptides, enhancement of the gut barrier function, and modulation of the immune response (Ghannoum et al., 2021). This yeast has been shown to stimulate the production of anti-inflammatory cytokines while suppressing pro-inflammatory responses (Khoury et al., 2018). Such immunomodulation is particularly relevant in the context of inflammatory bowel diseases, where an overactive immune

system plays a central role (Khoury et al., 2018). Studies show that *S. boulardii* increases the secretion of immunoglobulin A. Immunomodulation is implemented by *S. boulardii* through the interaction of mucosal dendritic cells (Buts et al., 1990). The potential applications of *S. cerevisiae* var. *boulardii* extend beyond gastrointestinal health. It's remarkable safety profile (Fijan, 2014), with rare reports of adverse effects (Feizizadeh et al., 2014), and further solidifies its status as a valuable probiotic.

1.3. *Alkalihalobacillus clausii*

Alkalihalobacillus clausii, previously known as *Bacillus clausii*, has been the subject of extensive research in the treatment of acute and infectious diarrheal diseases, including acute and chronic diarrhea, acute community-acquired diarrhea (ACAD), antibiotic-associated diarrhea (AAD), and *Clostridium difficile*-induced diarrhea (CDID) across different regions (Hamid et al., 2019; Ianiro et al., 2018; European Medicines, 2017). This probiotic is deemed safe for children, adults, and the elderly, and is available over the counter as products like Enterogermina® (Wu et al., 2019). Characterized by an average genome size ranging from 4,197,324 to 4,598,557 base pairs and a GC content between 42.8% and 44.75%, *Alkalihalobacillus clausii* is notable for its ability to form spores, high alkaline protease production, and production of antimicrobials like clausin, a type A lantibiotic (Bouhss et al., 2009; Senesi et al., 2001). It enhances gut health by improving the digestive microenvironment, modulating gut microbiota, and regulating the host immune system (Duysburgh et al., 2023).

Enterogermina® contains four poly-antibiotic resistant strains of *A. clausii* (O/C, SIN, N/R, and T), which reduced the incidence (by 39%) and duration of diarrhea in patients undergoing *H. pylori* eradication therapy (Plomer et al., 2020). Other pathogens like *Clostridium perfringens, Staphylococcus aureus, Klebsiella oxytoca, Candida* spp*., Pseudomonas* spp*., and Salmonella* spp. can occasionally disrupt intestinal microbial balance. Antibiotics such as β-lactam, lincomycin, cephalosporins, and macrolides are commonly used to control these pathogens, though they may cause adverse effects like infectious or antibiotic-associated diarrhea (Guo et al., 2019). A key feature of *Alkalihalobacillus clausii* is its spore-forming ability, allowing it to withstand extreme

environmental conditions including high temperatures, desiccation, osmotic pressure, and gastrointestinal challenges like gastric acid, pepsin, pancreatin, digestive enzymes, bile, and mucins (Khokhlova et al., 2023).

The probiotic's efficacy varies among strains and depends on factors like dosage and the severity of clinical conditions. Generally considered safe for human consumption, it is listed by the European Food Safety Authority (EFSA) as a bacterium with a Qualified Presumption of Safety (QPS) (Maity & Gupta, 2021). *Alkalihalobacillus clausii* strains possess intrinsic antibiotic resistance genes in their chromosomal DNA, making them stable and non-transferable (Castro et al., 2020). This resistance is beneficial as it allows these probiotics to be used alongside antibiotics to restore microbiota in gastrointestinal disorders, including antibiotic-associated diarrhea. The clinical benefits of various *A. clausii* strains can differ based on their ability to survive and proliferate in the gastrointestinal tract, adhere to epithelial cells, modulate the immune system, and adapt to the host (Srinivas, 2020).

A study by Maity et al. examined the clinical efficacy and safety of the probiotic *A. clausii* 088AE (MCC 0538) in alleviating antibiotic-associated diarrhea and related symptoms in pediatric, adolescent, and adult populations. This specific strain, *Alkalihalobacillus clausii* 088AE, with a genome size of 4,598,557 base pairs and a GC content of 44.74%, was used in the clinical trial (Maity & Gupta, 2021). The study evaluated the frequency of diarrhea, severity of associated symptoms, and stool consistency in the intervention group compared to the control group. *Alkalihalobacillus clausii* shows great potential as a probiotic for managing various gastrointestinal conditions, and your research adds valuable insights into its clinical applications and safety.

1.4. Human Microbiota

The human microbiota, a varied group of microorganisms found mainly in anatomical locations including the gastrointestinal system, mucosa, skin, respiratory tract, urogenital tract, and mammary gland, creates a complex ecosystem with the host (Aarnoutse et al., 2019). This symbiotic relationship, established from birth, significantly

affects physiological processes such as metabolism, immunity, and neurology (Sarma et al., 2018). The microbiota adapts to particular niches influenced by genetics, diet, and environmental exposures (Jayarathne et al., 2019).

Dysbiosis, defined as an imbalance in microbial composition, may arise due to various factors such as ageing, nutrition, lifestyle, hormonal alterations, inherited genes, and underlying medical conditions leading to health implications (Meng et al., 2017). The gut has a crucial role in this regard. Probiotics utilize microbiota associations to amplify advantageous elements, underscoring the significance of comprehending microbiota for precise health interventions. Advanced sequencing technologies have transformed understanding, demonstrating the assorted constitution and crucial function in metabolic, nutritional, and immunological pathways (Jiang et al., 2021).

The gut microbiota, highly concentrated in the gastrointestinal tract, remains stable throughout life, with diet, genetics and the innate immune system influencing its composition. The concept of enterotypes categorises the human gut microbiome and provides stable solutions at the metabolome level (Arumugam et al., 2011). The gut microbiota plays a vital role in nutrient metabolism, synthesising vitamins, catabolising cholesterol and participating in immune reactions. The development of the host microbiota influences both the immune system and the brain (Zuo et al., 2023). Dysbiotic conditions are temporarily present, indicating the microbiota's resilience (Meng et al., 2017). The intestinal microbiota, consisting of over 1200 bacterial species in the colon, has a significant impact on gut homeostasis and, consequently, influences the host's behaviour, cognitive functions, and health (Qin et al., 2010).

The human gastrointestinal tract accommodates more than 100 trillion microbes, surpassing the gene count of the human genome. Microbial composition varies across anatomical sites. The stomach hosts *bacilli, catenabacteria, enterococci* and *lactobacilli* (Roberfroid et al. 2010). The duodenum features *lactobacilli, streptococci, veillonellae, staphylococci, actinobacilli*, and yeasts, with microbial load increasing from the duodenum to the ileum (Booijink et al., 2010). The large intestine contains a diverse microbiota (10^7-10^{12} CFU/ml), of which over 80% are unculturable *in vitro* (Eckburg et al., 2005). Factors such as pH, peristalsis, and nutrient availability have a significant impact on microbiota diversity, which is crucial for maintaining "normobiosis" and gut homeostasis. The gut microbiota regulates energy levels, metabolism, drug neutralization, intestinal motility, and immunity, and also acts as a barrier against pathogens (Sommer&

Backhed, 2013). Inflammation caused by impaired intestinal mucosal immunity may contribute to various illnesses (Blottiere et al., 2013).

The human body has ten times more microbial cells (10^{14}) than human cells, and the gastrointestinal track has the largest concentration of these cells. This fact has been known for more than thirty years (Savage, 1977). The intestinal microbiota substantially contributes to metabolic, nutritional, physiological and immunological processes. It extracts energy from indigestible dietary components and protects against pathogens, while also participating in gastrointestinal functions and impacting the immune system of the mucosa (Carmona-Gutierrez et al., 2022). It is crucial to comprehend the diversity and makeup of the gut microbiota. Innovative molecular techniques provide important new understandings of its phylogenetic and functional properties (Fernandez-Rozadilla et al., 2021; Trang-Poisson et al., 2020; Diakite et al., 2019). The complex relationship between human health and the gut microbiota has been brought to light by recent studies. Changing the gut microbiota offers potential ways to improve health outcomes, particularly when done using probiotics (FAO/WHO, 2002).

1.5. Ensuring Viability and Efficacy of Probiotics

The increasing popularity of probiotic products has highlighted the importance of ensuring the viability of these live microorganisms. Probiotics must survive many phases of manufacture, storage, and ingestion in order to boost the host's health. The surge in the demand for probiotic products, specifically those containing lactic acid bacteria (LAB), has been fueled by the increased knowledge of their possible health advantages (Mattison et al., 2020; Jung et al., 201; (Noda et al., 2020; Watanabe et al., 2021; Yang et al., 2016). This trend has not only led to a diverse range of probiotic products but has also emphasized the importance of reliable methods to ensure the survival of probiotics in an expanding market of traditional fermented foods and advanced supplements (Kolaček et al., 2017).

Probiotics are a unique type of product, distinguished by their composition of live microorganisms, which have been defined by the FAO/WHO as having the ability to confer health benefits when consumed in sufficient quantities (FAO/WHO, 2002). This

particular characteristic requires strict assessments of viability, as mandated by regulatory bodies and measured in CFU's through plate count enumeration methods. Due to their recommended daily dosage of 10^9 CFU, determining the health effects of probiotics becomes even more complex (Vitetta et al., 2014). However, viability is a multifaceted parameter going beyond the capacity to establish colonies. In order to acquire a thorough picture of the probiotic state, a detailed study of a larger set of features is necessary in order to determine viability (Hansen et al., 2018). The challenge intensifies when considering the intricate compositions of probiotic products, often incorporating multiple strains and additional active ingredients, which further complicates viability analysis and demands a more intricate evaluation framework. A pivotal facet of the definition of probiotics hinges on the assumption that a substantial proportion of these microorganisms should remain viable during their passage through the gastrointestinal tract (GIT), where they must contend with strong stimulants like stomach acid and bile (Sánchez-Vega & Aryana, 2012).

The efficacy of probiotics in conferring health benefits is intricately tied to achieving a critical concentration of viable microorganisms, often regarded as 10^6 CFU/ml in the colon and 10^8 CFU/g in the small intestine (Minelli and Benini, 2009). Despite these considerations, current regulatory practices often fall short of mandating tolerance analyses against GIT stressors. When performed, such analyses are typically confined to the research and discovery phase rather than during the process of creating the probiotic product that will eventually be sold to customers (Fiore et al., 2020). This underscores a critical gap in the current regulatory framework where survival post-exposure to bile and gastric acid can significantly vary, often by several log units, contingent upon formulation intricacies, freeze-drying methods, and storage conditions (Chen et al., 2019; Salman, 2022; Wang et al., 2023). Intriguingly, the probiotic landscape encompasses a myriad of criteria, including safety, technological, functional, and physiological characteristics, all of which play a pivotal role in the selection of suitable strains for incorporation into various products (Carrillo-Lopez et al., 2021; Ballini et al., 2023).

The selection criteria encompass a wide range, from safety considerations such as origin, pathogenicity, and infectivity properties to technological aspects including genetic stability, phage resistance, and desired viability during processing and storage (Jung et al., 2019). Functional criteria focus on resistance to acid, bile, and pancreatic enzymes, as well as adhesion to mucosal surfaces (Jung et al., 2019). Additionally, physiological

criteria address documented health benefits such as lactose metabolism, immunomodulation, antagonistic activity, anticholesterolemic effects, antimutagenicity, and anticarcinogenic properties (Anadon et al., 2015).

The effectiveness of probiotic bacteria in food products relies on their ability to remain viable throughout their shelf life. To ensure probiotic foods deliver positive health effects, they must maintain a minimum viable and active cell count per gram or milliliter at the time of consumption. While there is no universally recommended standard for live probiotic bacterial counts, guidelines like the Codex standard on fermented milk suggest a minimum of 10^6 cfu/g of product (Gustaw et al., 2021). However, determining a specific level of probiotics for all beneficial effects is challenging, and recommendations often assume a daily consumption of 100g of probiotic product (Maselli & Hekmat, 2016). Consequently, the generally accepted minimum necessary concentration of probiotic bacteria is 10^6 cfu/g upon consumption, based on the assumption of daily consumption of 100g of probiotic product (Gustaw et al., 2021).

Ensuring the viability of probiotics in food is crucial for achieving their beneficial health effects. Maintaining an adequate count of probiotic survival during storage involves various factors, including strain selection, production conditions, and the method of incorporation into food matrices. Probiotic cultures, often delivered as food supplements or incorporated into various food and beverage matrices, encounter stress conditions such as temperature, oxygen, and relative humidity, which can impact their viability (Hossain et al., 2020).

The choice of strain is a critical factor in the effectiveness of probiotic cultures and depends on their adaptability to the specific food matrix and the physiological environment of the human intestine (Villamor-Martinez et al., 2017). The method of incorporation, whether through direct inoculation or freeze-drying, also significantly affects viability. Each approach presents its own challenges, requiring careful consideration of thawing parameters, rehydration conditions, and subsequent effects on viability. The processing steps during food manufacture are essential determinants, with some technological procedures posing challenges to probiotic survival.

Strategies to mitigate viability losses during processing include modifying the food matrix, adjusting pH conditions, adding antioxidants and growth factors, using nontoxic ingredients, and adapting food processing steps, such as lowering temperatures, modifying fermentation parameters, and subjecting cells to sublethal stresses (Betoret et al., 2020). Additionally, the timing of incorporating probiotic cultures during food

processing is crucial (Lin et al., 2022). The stages of food production, storage, and inoculation, including the production and storage of dried probiotic cultures and the preparation of rehydration media, significantly influence their viability (Korcok et al., 2018). Each stage presents specific challenges and opportunities that require careful attention.

Freeze-drying, a commonly used technique for preserving probiotic cultures, requires meticulous optimization of parameters such as freezing rate, cryoprotectant type and concentration, and drying rate (Chen et al., 2019). These parameters affect the viability and stability of probiotic cultures during storage and rehydration. Proper storage conditions post-manufacture are also critical for maintaining probiotic viability. Probiotics are often exposed to conditions such as temperature fluctuations, moisture, and oxygen during storage, which can compromise their viability. Therefore, implementing suitable storage conditions, such as refrigeration or freeze-drying, is crucial for maintaining probiotic viability throughout the product's shelf life. Moreover, probiotic viability faces an added layer of complexity due to the dynamic nature of the gastrointestinal tract (GIT) environment.

Surviving exposure to bile and gastric acid is a crucial variable, and probiotics' ability to withstand these harsh conditions significantly affects their efficacy in providing health benefits (Shi et al., 2016). The survival of probiotics during GIT transit is also influenced by the type of food matrix used for delivery. Food matrices can act as protective barriers against harsh GIT conditions and therefore, the choice of food matrix is crucial in determining the fate of probiotics (Dimidi et al., 2019).

Dairy products have been traditionally used as probiotic carriers due to their composition and inherent properties, but there is a growing interest in exploring non-dairy options to cater to specific populations and increase product versatility. Additionally, when formulating multi-strain probiotic products, the coexistence and interactions between strains must be considered. These interactions can impact the overall viability and functionality of the probiotic consortium, highlighting the need for a thorough understanding of strain interactions and compatibility (Forssten et al., 2020).

1.6. Microencapsulation

1.6.1. Microencapsulation Techniques

The challenges encountered by researchers and industries in ensuring high levels of viable probiotic bacteria in non-dairy functional foods constitute a pivotal aspect of integrating probiotics into such products. The preservation of probiotic viability throughout processing, storage, and transit through the digestive tract is imperative for their advantageous functionalities. Various factors, encompassing temperature variations, low pH, water activity, additives, antimicrobial substances, and digestive enzymes, pose impediments necessitating effective preservation strategies (Klojdová et al., 2023). The microencapsulation technique has emerged as a promising solution to safeguard probiotics, providing protection during processing, storage, and challenges encountered in the digestive system. . The main benefits of microencapsulation process can be illustrated in Figure 1.

Microencapsulation, characterized as a process entailing the entrapment of an active agent within a suitable wall material, addresses the challenges associated with probiotic viability comprehensively (Plessas, 2021). By this method, probiotic bacterial cells are prevented from undergoing harm and their release within the gastrointestinal system is regulated as they are encapsulated in food-grade encapsulating chemicals (Kavas et al., 2022). Nevertheless, the size of bacterial cells poses a significant hurdle to the application of nanotechnology, constraining both cell loading and viability maintenance (Razavi et al., 2021). The success of microencapsulation hinges on various parameters, including physicochemical properties, process conditions, particle size, and storage conditions, underscoring the importance of ongoing research in this domain (Jyothi et al., 2010).

Despite strides in microencapsulation techniques, persistent challenges include maintaining aseptic conditions, addressing storage leakage, and enhancing thermal resistance. Ongoing efforts involve incorporating diverse polymers, cryoprotectants, antioxidants, and prebiotics into the encapsulation matrix (Rodrigues et al., 2020). Innovative approaches, such as double coating, further underscore the commitment to

improving probiotic stability (Pupa et al., 2021). Additionally, the critical evaluation of microencapsulation products through in vivo studies remains essential, urging further exploration involving animal models and human trials.

Bacterial viability, a critical determinant of probiotic efficacy, is contingent upon environmental conditions, food matrix characteristics, and the presence of additives (Shori, 2016). Microencapsulation aims to address viability issues, necessitating careful consideration of all influencing factors during probiotic formulation development. Challenges related to oxygen exposure, humidity, temperature, pH, and food additives must be navigated to ensure probiotic survival from production to consumption (Terpou et al., 2019).

MICROENCAPSULATION

Improved Viability (Palanivelu et al., 2021; Etchepare et al., 2019; Bosnea et al., 2014)

Improved Bioavailability (Mhatre & Gurav, 2020; Pereira-Caro et al., 2015)

Enhanced Stability in Formulations (Barajas-Álvarez et al., 20203; Ying et al., 2016; Champagne et al., 2015)

Controlled Release in the Gut (Huang et al., 2021; Basu et al., 2018; Cook et al., 2012)

Protection Against Harsh Conditions (Liu et al., 2019; Rokka & Rantamäki, 2010; Anal & Singh, 2007)

Extended Shelf Life (Gullifa et al., 2023; Anekella & Orsat, 2014)

Masking of Flavor and Odor (Calderón-Oliver & Ponce-Alquicira, 2022; Sanguansri & Ann Augustin, 2010)

Figure 1: Main benefits of microencapsulation

The ongoing pursuit of improved stability and colon adhesion of probiotics emphasizes the imperative for effective encapsulation systems. Encapsulation, whether achieved through spray drying, emulsification, extrusion, electrospraying, or freeze-drying, serves as a physicochemical or mechanical technique to shield probiotic cells

from environmental fluctuations (Agriopoulou et al., 2023). Among the diverse probiotic encapsulation technologies, emulsion techniques stand out for their cost-effectiveness, simplicity, and mild process conditions, facilitating the production of microparticles (Camelo-Silva et al., 2022). It is essential to choose a suitable encapsulation approach depending on the sensitivity of the probiotic species, preferably using non-toxic solvents and mild, non-aggressive processes. The encapsulation of probiotics, along with other active ingredients, has become standard practice in the food industry, highlighting the versatility and significance of encapsulation techniques. The subsequent paragraphs delineate the principal techniques employed for the encapsulation of probiotic cells

1.6.1.1. Emulsion Method

The emulsion technique stands as a pivotal approach in the realm of probiotic microencapsulation, offering a methodology to safeguard probiotic cells within protective matrices. At its core, the emulsion method involves the dispersion of probiotic cells within a continuous phase of a suitable carrier material (Rodrigues et al., 2020). This dispersion is achieved through rigorous high-shear mixing or homogenization, ensuring a consistent distribution of probiotic cells throughout the carrier (Jamshidi et al., 2020). The significance of the choice of carrier material cannot be overstated. It profoundly influences the properties of the resultant microcapsules, including but not limited to stability, viability, and the release dynamics of the encapsulated probiotics (Koç et al., 2015). Among the commonly employed materials for this purpose are alginate, carrageenan, and pectin.

In a typical emulsion process, a probiotic suspension, often mixed with prebiotics and polymers, serves as the dispersed phase (Camelo-Silva et al., 2022). This is combined with a continuous phase, usually a vegetable oil, wherein an emulsifier stabilizes the resulting mixture (Camelo-Silva et al., 2022). The use of a solidifying agent, such as calcium chloride, is a crucial next step that solidifies the water-soluble polymer and creates gel particles (Lu et al., 2019). A flow chart describing encapsulation by emulsification process can be seen in Figure 2.
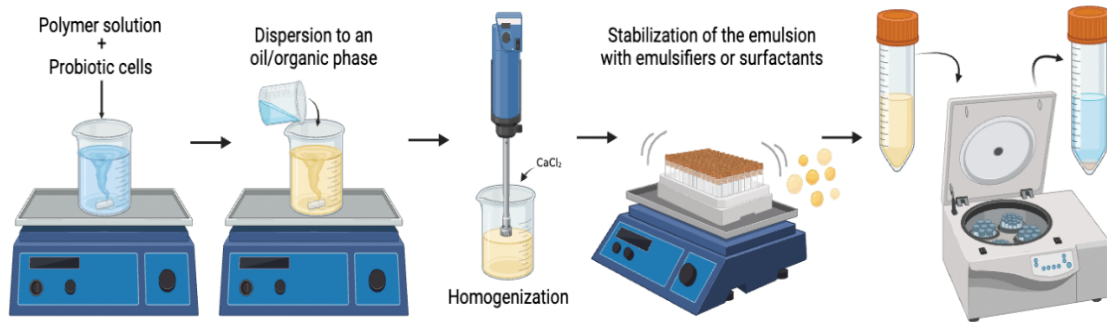
Figure 2: Flow chart for the emulsification process used to encapsulate probiotics

Emulsions can manifest in various forms based on the interactions between the phases. For example, based on the dispersed phase, simple emulsions can be classified as either oil-in-water (O/W) or water-in-oil (W/O). Further complexity arises with the formation of multiple emulsions, exemplified by structures like water-in-oil-in-water (W/O/W) (Burgain et al., 2011). These configurations offer tailored strategies to enhance the protection of encapsulated probiotics, particularly leveraging the hydrophilic nature of these cells (Wang et al., 2020).

While emulsification provides a promising encapsulation avenue, challenges such as the broad size distribution of the produced microparticles and potential decreases in probiotic viability over extended storage durations necessitate further refinements (Rathore et al., 2013). Techniques like layer-by-layer (LbL) coating, where additional protective layers are applied to the microcapsules, have emerged to address these concerns (Li et al., 2023). Moreover, processes like freeze-drying or spray-drying can augment the survival rates of probiotics post-encapsulation (Dutta et al., 2018).

1.6.1.2. Spray Drying Method

Spray drying is a pivotal technique in probiotic microencapsulation, bridging the gap between liquid formulations and dry powders, especially within the food industry (Sharma et al., 2022). This method involves the rapid drying of a liquid or slurry using hot gas, transforming it into a powdered form. Spray drying effectively encapsulates both

the polymer matrix and the delicate probiotic live cells, showcasing its versatility. Polymers such as gum arabic and starch have been observed to form spherical microparticles during the drying process (Bucurescu et al., 2018). However, the use of high temperatures in spray drying can compromise bacterial viability, underscoring the need for thermostable carriers (Hao et al., 2021). Spray drying process for encapsulation purposes can be shown in Figure 3.

The co-encapsulation of bioactive ingredients, particularly prebiotics and probiotics, is essential for the effectiveness of spray drying. The process involves dispersing the ingredients in a solution containing a carrier, then atomizing the mixture in a heated-air chamber. The resulting dried particles encapsulate the bioactive components within a wall material. Various factors, including spray drying conditions and dispersion properties, influence encapsulation efficiency and particle size (Shamaei et al., 2017). Spray drying is a rapid process that subjects materials to high temperatures for only a few seconds, making it suitable for encapsulating heat-sensitive ingredients such as probiotics (Liu et al., 2015). Recent studies have highlighted the potential of spray drying, demonstrating significant improvements in probiotic stability and viability, especially when combined with polysaccharides (Bustamante et al., 2020; Tao et al., 2019).

The physical dimensions of the particles resulting from spray drying determine its utility. Microparticles are predominantly produced by traditional spray dryers, while nano spray dryers produce particles at the nanoscale (Sosnik et al., 2015). The latter, facilitated by a piezoelectric vibrating mesh, offers enhanced bioavailability. However, co-encapsulating probiotics is constrained due to their larger size (Arpagaus et al., 2018). However, spray drying faces challenges in terms of effectiveness. The encapsulation outcome can be influenced by various factors, including the probiotic strain's characteristics, drying parameters, carrier materials, and storage conditions (Ermis, 2022). To mitigate these challenges, strategies such as adjusting drying parameters, incorporating protective agents like trehalose, and utilizing appropriate wall materials such as carbohydrates and proteins have been proposed (Obradović et al., 2022; Nunes et al., 2018; Rokka & Rantamäki, 2010).
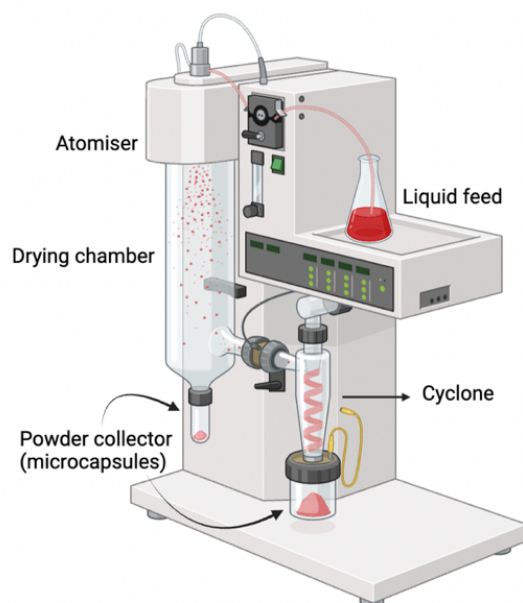
Figure 3: The spray drying process used to encapsulate probiotics (Created with BioRender.com)

1.6.1.3. Extrusion Method

The extrusion technique is widely used for microencapsulation, particularly for encapsulating probiotic microorganisms in hydrocolloid gel matrices. This method primarily employs hydrocolloids like alginate and carrageenan as protective coatings for probiotics, shielding them from external adversities during storage (Ta et al., 2021; Muhardina et al., 2018). The extrusion process involves preparing an aqueous hydrocolloid solution and incorporating concentrated microorganisms into it. This mixture is then passed through a nozzle, forming droplets that descend into a hardening solution, typically a calcium chloride ($CaCl_2$) solution (Li et al., 2019). The size of the resulting capsules is influenced by several factors, including the diameter of the orifice, the distance between the nozzle and the hardening solution, and the viscosity of the hydrocolloid-microorganism blend (Anal & Singh, 2007).

The extrusion technique has a significant advantage in preserving probiotic viability. The extrusion method has demonstrated impressive survival rates of probiotics, ranging from 85% to 90%, even under challenging conditions such as exposure to gastric

acid and bile. This outperforms various other encapsulation methods (Das et al., 2014; Cook et al., 2012). Additionally, extrusion avoids elevated processing temperatures, ensuring a higher probiotic survival rate. However, extrusion, like any method, has limitations. One primary concern is the bead size, which typically ranges from 2 to 5 mm and may not be optimal for certain applications (Burgain et al. 2011). Additionally, while feasible for smaller-scale operations, scaling up presents challenges due to the slower rate of particle formation (Burgain et al., 2011). Innovative approaches, such as integrating multiple-nozzle systems and advanced atomization techniques, are being explored to overcome scalability issues (Frakolaki et al., 2021). Figure 4 shows the extrusion process used for encapsulating probiotics.
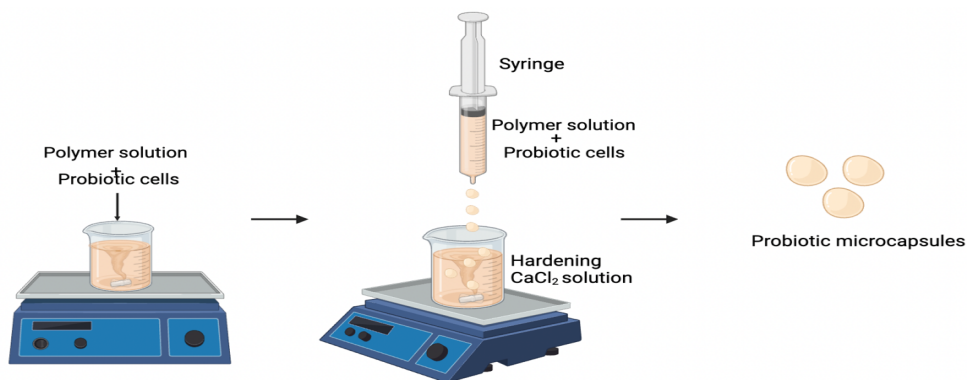
Figure 4: Flow chart for the extrusion process used to encapsulate probiotics,

1.6.1.4. Electrospraying – electrospinning

Electrospraying and electrospinning are electrohydrodynamic processes that use high voltage electric fields to atomise cell solutions, resulting in the deposition of either particles or fibres. The setup includes a high-voltage source (1–30kV), a needle or capillary, a syringe pump, and a grounded collector (Anu Bhushani & Anandharamakrishnan 2014).

Electrospinning and electrospraying are two methods used to transform cell solutions and liquids into fibers and droplets, respectively, *via* electrostatic forces

(Phuong Ta et al., 2021; Gomez-Mascaraque et al. 2016). The outcome of these methods depends on the concentration of the solution, with higher concentrations yielding fibers and lower concentrations resulting in droplets (Ghorani & Tucker, 2015). These methods are efficient, adaptable, and can produce particles ranging from micron to nano scales. Microbial cell integrity is maintained and food-grade solvents are used, however, solution dripping may occur under non-optimal conditions (Gomez-Mascaraque et al., 2016; Anu Bhushani & Anandharamakrishnan, 2014). Techniques are described in Figure 5.

Electrospraying involves electrifying a conductive polymer solution and directing it towards a collector. This technique, which is known for its simplicity, is becoming increasingly popular for encapsulating substances such as probiotics. However, it can sometimes produce limited particles and require meticulous optimization (Phuong Ta et al., 2021; Zaeim et al., 2018; Tapia-Hernandez et al., 2015).
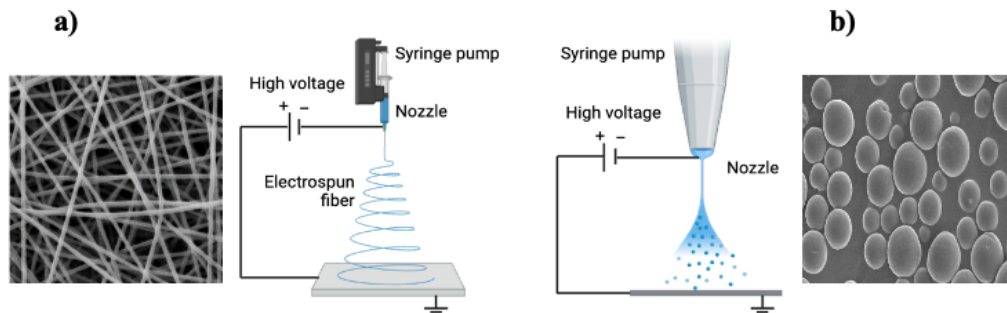
Figure 5: Flow diagram of the process of encapsulation by electrospraying and electrospinning a) Electrospinning process and electrospun fiber image under SEM, b) Electrospraying process and electrospray capsules image under SEM

1.6.2. Coating Materials Used in Microencapsulation

Several recent studies (Rehman, Tong et al. 2019; Singh et al. 2018; Zaeim et al. 2019) have demonstrated the effectiveness of biocompatible and food-grade wall materials in enhancing the protection of bioactive ingredients against digestive challenges. Edible delivery systems utilize shells, coatings, carriers, or membranes as

essential barriers. This article examines the important roles of polysaccharides, proteins, lipids, and minerals as coating materials and their use in co-encapsulating probiotics and prebiotics. The choice of suitable encapsulating agents is crucial for the success of microencapsulation. Combining alginate with other materials to form multiple layers that overcome inherent limitations can significantly improve its effectiveness. Singh et al. (2018) assert the significance of meticulous material selection for achieving successful microencapsulation.

Encapsulation yield, a pivotal metric influenced by factors such as agitation rate, encapsulating materials, and probiotic strain, is imperative for achieving success. Materials such as sodium caseinate, sodium alginate, gelatin, and Arabic gum, as well as innovative combinations involving pectin with rice bran or inulin, exhibit high encapsulation yields. This positively impacts survival rates during storage and exposure to gastrointestinal conditions (Zaeim et al., 2019). It is crucial to have a comprehensive understanding of the properties and interactions within food-grade wall materials for successful co-encapsulation. Polysaccharides, proteins, lipids, and minerals each play distinctive roles and offer a spectrum of benefits. It is essential to thoroughly investigate and understand these complex interactions, supported by literature evidence, to advance the field of encapsulation for optimized probiotic delivery and associated health benefits.

Polysaccharides such as (Graff et al., 2008), pectin (Surolia et al., 2023), carrageenan (Nunes et al., 2018), gums (Boonanuntanasarn et al.,2018; Arslan-Tontul & Erbas, 2017), starch (Arslan et al., 2015) and alginate (Ramirez-Olea et al., 2024; Singh et al., 2019) are widely employed to create amorphous glassy entities that provide essential support to conveyance structure walls. Alginate has been highlighted as an effective means of preserving probiotic integrity in noteworthy literature (Rehman, Tong et al. 2019). The use of polysaccharides in microencapsulation ensures the preservation of probiotic viability, thereby enhancing their functionality in various applications, including functional foods and pharmaceuticals. Polysaccharides are an excellent choice for the food industry due to their natural origin and safety. They provide a dependable and sustainable method for enhancing probiotic delivery and efficacy.

Animal-derived proteins, including collagen, gelatin, and whey proteins, as well as plant-derived proteins, such as gliadin and soy proteins, possess exceptional amphiphilic properties, superior emulsifying abilities, and remarkable gelation capacities (Xu et al., 2023). These properties make them the perfect choice for safeguarding encapsulated viability.

Table 1 demonstrates the significant protection and controlled release attributes of encapsulated probiotics using pioneering studies that employed pea protein isolate (Arslan et al., 2015), whey protein isolate (Alehosseini et al., 2019; Arslan et al., 2015; Hébrard et al., 2010), and inulin (Ramirez-Olea et al., 2024; Fratianni et al., 2014) as wall materials. Proteins in microencapsulation offer the added advantage of being a natural and food-grade material, rendering it appropriate for application in the food and pharmaceutical sectors.

Table 1: Probiotic strains, methods and coating materials used in microencapsulation

Probiotic Strain	Encapsulation Method	Encapsulation Materials	References
Bacillus clausii	Spray Drying	Maltodextrin, alginate, inulin	Ramirez-Olea et al. (2024)
Bacillus clausii, Saccharomyces boulardii	Extrusion	Pectin	Surolia et al. (2023)
Lactobacillus acidophilus, Bacillus clausii, Saccharomyces boulardii	Hydrogel	Sodium alginate and gelatin	Du Le , H., & Son Trinh, K. (2018)
Saccharomyces boulardii	Spray Drying	WPC, maltodextrin, Gelatin, modified starch, pea protein isolate and gum Arabic	Arslan et al. (2015)
Saccharomyces boulardii	Emulsification	Alginate, inulin, and xanthan gum	Fratianni et al. (2014)

(cont. on next page)

Table 1 (cont.)

Saccharomyces boulardii	Extrusion	Alginate / chitosan	Graff et al. (2008)
Saccharomyces boulardii	Extrusion/cold gelation	Whey protein isolate, Alginate	Hébrard et al. (2010)
Saccharomyces cerevisiae DSY-5	Lyophilisation	Guar gum	Boonanuntanasarn et al. (2018)
Saccharomyces boulardii, Bifidobacterium bifidum BB-12, *Lactobacillus acidophilus* LA-5,	Spray drying / Spray chilling	Gum arabic, β-cyclodextrin / hydrogenated palm oil	Arslan-Tontul & Erbas (2017)
Lactobacillus rhamnosus	Spray and freeze-drying	Maltodextrin and gum Arabic	Mishra and Athmaselvi (2016)
Lactobacillus plantarum	Extrusion	Gum Arabic and Sodium alginate	Sandoval Mosqueda et al. (2019)
Lactobacillus acidophilus	Spray drying	Carrageenan	Nunes et al. (2018)
Lactobacillus casei	Electro-encapsulation	Whey protein	Alehosseini et al. (2019)
Lactobacillus gastricus	Extrusion	Skimmed milk and alginate	Singh et al. (2019)
Bifidobacterium animalis subsp. *lactis* Bb12	Electrospinning	Pullulan	López-Rubio et al. (2012)
Bifidobacterium lactis	Electrospinning	Whey protein concentrate (WPC) and pullulan	Lopez-Rubio et al. (2009)
Bifidobacterium breve	Emulsion and/or spray-drying	Milk fat and/or denatured whey proteins	Yasmin et al. (2018)

Lipids, both polar (e.g. monoglycerides and phospholipids) and non-polar (e.g. cholesterol and triglycerides), are highly effective wall materials for microencapsulation (Rehman, Tong et al. 2019). This process entails enveloping probiotic cells with a lipid-based shell to shield them from external factors, such as pH fluctuations, oxygen exposure, and storage conditions. The lipid matrix shields the probiotics from harsh environmental conditions and enhances their stability during processing and storage. Emulsion methods that use oils such as palm and palm kernel oil have successfully improved survival rates during exposure to simulated gastrointestinal conditions. As Zaeim et al. (2019) state, literature supports the effectiveness of lipid-based encapsulation in strengthening probiotic resilience.

Incorporating minerals, such as magnesium oxide (MgO) in alginate-gelatin microgels (Du Le, H., & Son Trinh, K., 2018) or antacid agents in alginate microgels, is a highly effective approach to enhance stability and viability. Studies (Rehman, Tong et al. 2019) have demonstrated the potential of these mineral-based techniques in protecting probiotics during their passage through the gastrointestinal tract.

1.7. Functional Foods

Functional foods, designed or modified with the specific intention of providing health benefits beyond traditional nutritional concepts, is a term used to describe foods that, when consistently ingested in suitable nutritional proportions, have the potential to have positive health impacts (Gul et al., 2016). This category encompasses a range of enriched, supplemented or fortified foods that, through the incorporation of specific bioactive compounds or active ingredients, contribute to supporting the general health of individuals, reducing the risk of disease or addressing specific health conditions (Wang & Bohn, 2012).

Functional foods are typically rich in antioxidants, probiotics, prebiotics, omega-3 fatty acids, vitamins, minerals, fibre and other bioactive components (Arshad et al., 2021). Antioxidants, found in fruits and vegetables such as strawberries, blackberries and spinach, can protect the body from oxidative stress caused by free radicals, reducing cell damage and preventing inflammation. Probiotics, found in products such as yoghurt, kefir

and fermented foods, are 'good' bacteria that support gut health. Omega-3 fatty acids, particularly from fish oil, can contribute to heart health, improve brain function and effectively fight inflammation. Functional foods, which contain vitamins and minerals, generally play a role in regulating bodily functions, providing energy and boosting the immune system. Consumption of functional foods can be approached as a dietary strategy aligned with an individual's specific health goals. However, the effects of these products may vary from person to person, which underlines the importance of consulting a healthcare professional. In addition, careful consideration of ingredients, calorie content and recommended intakes is crucial for consumers to ensure a balanced nutritional profile.

The recent surge in interest in functional foods can be attributed to a number of factors, including increased health awareness, changes in food legislation and a surge in scientific research into the relationship between diet and well-being. The growing popularity of functional foods is also supported by the food industry's continued efforts to develop and diversify such products. Globally, a variety of terms such as functional foods, nutraceuticals, pharmaceutical foods, decorator products, pharmabiotics, vitabiotics and others are used to define natural health-promoting foods (Chhikara et al., 2022). When categorised separately, these terms may include medical foods, dietary supplements, fortified foods, and botanicals, which encapsulate similar regulators (Alamgir & Alamgir, 2017). Functional foods can include various fortified and enriched elements that, when effectively consumed within a modified diet, provide health benefits distinct from essential nutrient elements (El Sohaimy, 2012). As a result, novel food formulations have been developed to address specific needs and physiological concerns, with snack bars being one of the products that have emerged from these formulations (Boukid et al., 2022).

1.7.1. Functional Food Trend: Integration of Probiotics into Snack Bars

Certain food matrices are more suitable for delivering probiotics. The food carrier also affects bacterial sensitivity to harsh gastrointestinal conditions such as acidity, bile, and various enzymes, as well as their adhesion capability to intestinal epithelial cells and

immunomodulatory properties (Bengoa et al., 2018). The buffering capacity of milk and milk fat ensures the survival of probiotics during processing and storage in dairy products like yogurt, cheese, and frozen fermented milk desserts. These products are therefore ideal and marketable carriers for probiotic bacteria (Granato et al., 2010). Among food products, yogurt is highly efficient as a probiotic vehicle, while ice cream, being rich in milk fat, is effective in enhancing microbial viability and acid tolerance (Gaba & Anand, 2023). However, the shelf-life of probiotic yogurt is limited due to oxidative stress experienced by probiotic bacteria (Deshwal et al., 2021). In several cultured dairy products, probiotic strains fail to meet the initial criterion for probiotics, which is to contain 'live microorganisms' at the time of consumption. This is because the product's acidity increases over time due to lactic acid-producing bacteria, making it intolerable for probiotic strains. Additionally, animal-based foods can be a source of antibiotic-resistant genes that may be transmitted to the gut microbiota. Commercially available antibiotic-resistant probiotic strains, primarily *Lactobacillus* and *Bifidobacterium*, have been detected in milk cultures, yoghurts, and cheeses (Wang et al., 2020).

Non-dairy food matrices are increasingly being explored as viable alternatives to dairy products for the distribution of probiotics and paraprobiotics. Health concerns associated with dairy products have led to a shift towards non-dairy foods such as fruits, vegetables, cereals, pulses, and soy, which can be effectively used as substrates to deliver the benefits of probiotics along with fiber to the consumer (Kumar et al., 2022). There are several reasons why individuals seek alternatives to probiotic and paraprobiotic dairy products. These include milk protein allergies, which affect 2-3% of children under three years of age, and lactose intolerance, the most common carbohydrate malabsorption disorder associated with low levels of the lactase enzyme, leading to an inability to digest lactose components (Gamirova et al., 2022; Park, 2021). Furthermore, milk-based foods have high cholesterol content and contain high levels of saturated fatty acids (Liu et al., 2019). The popularity of vegan or vegetarian diets is influenced by various factors, including the growing health awareness among the general population. Moreover, traditional milk and dairy products are receiving significant attention due to potential contaminants such as hormones, pesticides, and antibiotics (Welsh et al., 2019). These concerns are even more critical for the elderly, who may have multimorbidity and various risk factors. The recent surge in healthcare costs and life expectancy has created an increasing demand for new non-dairy functional foods that promote healthy eating and improve quality of life. Probiotics and paraprobiotics are versatile ingredients that can be

used in a variety of non-dairy functional foods, pharmaceuticals, supplements, and animal feeds (Barros et al., 2020). The industrial sector for functional foods containing probiotics is currently experiencing significant commercial interest and growing market shares.

Preparing a probiotic product presents several challenges related to microbial growth, survival, viability, stability, and functionality during food processing, storage, and consumption. However, with our expertise in probiotic cultures, we have successfully overcome these challenges and developed effective solutions for non-dairy products. Our probiotic cultures have been specifically formulated to ensure optimal stability and proliferation, even in non-dairy products. Trust us to deliver the highest quality probiotic products for your needs. Formulating non-dairy products, such as grain products, beverages, and candies, requires careful consideration of factors such as water activity, oxygen tension, and temperature (Ranadheera et al., 2020). This is particularly crucial for probiotic stability, which can be challenging to maintain during room temperature storage (Barajas-Álvarez et al., 2023). It is important to note that adding probiotics to fruit- and grain-based matrices is a more complex process compared to formulating dairy products. To address these drawbacks, paraprobiotics can confidently be incorporated as ingredients in non-dairy products. This is particularly useful in situations where probiotics may be compromised and unable to survive during processing and/or shelf life (Siciliano et al., 2021).Snack bars are an excellent alternative to functional foods containing probiotics. They are widely consumed by individuals who need a quick energy source due to a lack of time for proper meals (Skoczek-Rubińska & Bajerska, 2021). Depending on the ingredients used and their intended use, there are various types of bars such as energy bars, food bars, protein bars, fruit bars, cereal bars, granola bars, nut bars, and sports bars. These ready-to-eat mixtures are compact, versatile, and convenient sources of carbohydrates, protein, and fat (Ayad et al., 2021). With their natural ingredients, including grains, nuts, and fruits, snack bars are significant sources of bioactive phytochemicals. They are highly effective practical supplements that can help reduce malnutrition and micronutrient deficiencies (Hastaoğlu et al., 2023). Snack bars are incredibly functional and convenient food options readily available on the market. Consumers seek snack bars for various purposes, such as cereal bars for breakfast, protein-rich sports bars for pre-workout snacks for athletes, and protein bars that appeal to a broad audience, including children. Bars enriched with vitamins can be stored as snacks for the whole family (Hastaoğlu et al., 2023).

Functional foods can benefit all individuals or specific community groups, such as those based on age or genetic makeup. In addition to providing basic nutrition, functional foods have been shown to improve physical condition and reduce the risk of developing diseases (Granato et al., 2020; Peng et al., 2020; Diez-Gutiérrez et al., 2020). However, it is important to note that the production of fast food and snack foods should not overshadow the importance of functional foods in maintaining a healthy lifestyle. Bower and Whitten (2000) classify many of the products that consumers often prefer as 'snacks,' including mini pizzas, cakes, popcorn, breakfast cereals, and cereal bars. Although traditionally, snack bars have not been considered functional foods due to their poor nutrient compositions, there is a trend towards new types of snack bars that contain functional components. These functional snack bars are not just a tasty treat but also provide convenient and acceptable ready-to-eat options for consumers, making them a viable option for those looking for a quick and healthy snack. The potential for incorporating bioactive compounds into functional snack bars to provide health benefits is significant. For instance, Dimopolulou et al. (2023) conducted a study on creating a diabetic and plant-based snack bar using mushroom (*Coprinus comatus*) powder. The resulting product exhibited enhanced nutritional content, particularly in omega-3 fatty acids, fiber, and protein, while maintaining a stable shelf life. Incorporating raisins and 100% coffee into snack bars has been shown to provide high levels of protection against oxidative stress, substantial phenolic content, and satisfactory acceptability (Souza Lara et al., 2018). This innovative combination presents a novel alternative. The inclusion of bean flour resulted in increased protein, fiber, and antioxidant capacity in snack bars, highlighting its efficacy as a source of bioactive compounds (Ramírez-Jiménez et al., 2018). Singh et al. (2022) studied the effects of adding oat and banana peel powder to functional snack bars. Their research demonstrated that these additions significantly increased levels of protein, minerals, β-glucan, dietary fiber, essential amino acids, phenolics, and antioxidant activity. Thermogravimetric analysis confirmed that the active components remained stable even at high temperatures, further enhancing the functional properties of the snack bars. Zulaikha et al. (2021) showed that enriching snack bars with tilapia (*Oreochromis niloticus*) by-product powders significantly increased protein content and angiotensin-converting enzyme (ACE) inhibitory activity. The ACE inhibitory activity was further enhanced during the baking process, and all samples exhibited antibacterial activity against *Staphylococcus aureus*. Functional snack bars enriched with diverse bioactive compounds have great potential as innovative and health-

promoting alternatives in the realm of functional foods. These studies showcase the effectiveness of such bars in promoting health and wellness.

The primary aim of this study is to develop a probiotic bar that supports gastrointestinal health through the microencapsulation of probiotic microorganisms, specifically *Saccharomyces boulardii* and *Alkalihalobacillus clausii*, within a lupine protein-gelatin-trehalose complex. The research seeks to address key questions: Can the incorporation of microencapsulated probiotic microorganisms into the bar content create a new functional product that provides protective effects within the gastrointestinal environment post-consumption? Additionally, will it be feasible to maintain the microbial quality of this probiotic and functional bar product throughout its shelf life? By exploring these questions, the study aims to establish the probiotic bar as a potential candidate for a novel functional food product.

CHAPTER 2

MATERIAL AND METHOD

2.1. Materials

The specific strains used in this study, *Saccharomyces boulardii* CNCM-I745, was sourced in lyophilized form from a reputable pharmacy (Biocodex, Reflor® 250 mg) and *Alkalihalobacillus clausii* (N/R, O/C, SIN, T), the commercial preparation Enterogermina® contains *Alkalihalobacillus clausii* spores were used (10^9 cfu/mL).

All of the chemicals were purchased from Merck and Sigma. Microorganism growth media: Tryptic Soy Broth (Merck, Germany, Catalogue number: 105459), VRB Agar (Violet Red Bile Agar, Merck, Germany, Catalogue number: 101406), PDA (Potato Dextrose Agar, Oxoid, England, Catalogue number: CM0139), and YPDA (Agar 2% (w/v), Peptone 2% (w/v), Yeast Extract 1% (w/v), D-Glucose 2% (w/v). The droplet hardening agent used in the microencapsulation procedure is $CaCl_2$ (Applichem, Germany; Catalogue number: 141221.1210).

The source of the soybean lecithin was Alfasol in Turkey. Lupin seeds and sunflower oil were supplied from a local market. Ingredients of probiotic bar are gluten-free oats (Patiswiss, Gluten Free Oat Flakes), dates (Peyman, Bahçeden Hurma), peanut butter (Fellas, 100% Peanut Butter), dried figs (Carrefour, Bio Organic Dried Figs), walnuts (Peyman, Bahçeden Ceviz), flax seeds (Arifoğlu, Flax Seed Ground), cinnamon, and salt.

2.2. Methods

At the beginning of the microencapsulation procedure, lupin protein was separated and utilized as a coating material. The probiotic strains were individually microencapsulated in lupin protein isolate-xanthan gum-trehalose complex. Following that, the production and characteristics of the probiotic bars were evaluated.

2.2.1. Lupin Protein Isolation

2.2.1.1. Solvent Defatting of Lupin Flour

Lupine seeds (*Lupinus albus*) were ground to a fine powder (650 μm particle size) for degreasing according to the method described by sn They were sieved through a 250 mesh stainless steel sieve to obtain homogeneous particle sizes. After mixing the flour and hexane in a solvent ratio of 1:3 (w/v) for 30 minutes, the oil was dissolved, and the solvent layer was removed by siphoning it off. To get the most degreasing, the extraction was done twice more. The mixture was filtered, washed with new solvent, and allowed to dry at room temperature in a fume hood for the last stage of degreasing.

2.2.1.2. Preparation of Lupin Protein Isolates

With minor adjustments, the alkali solubilization and isoelectric precipitation method outlined by Snowden, Sipsas, and St. John (2007) was used to generate the lupine protein isolate. First, distilled water was combined with defatted lupine meal at a ratio of 1:10 (w/v). After adding 1 M of NaOH to the suspension, it was stirred for 90 minutes at 25°C. After centrifuging the suspension for 15 minutes at 4°C and 3500 rpm (using a Sigma Laboratory Centrifuge 6K15), the supernatant containing the isolated proteins was

collected. After adjusting the pH of the supernatant to 4.5 using HCl (1 M) to precipitate the proteins at their isoelectric point (pI), the proteins were separated by centrifugation at 3500 rpm for 15 minutes at 4 °C.

2.2.2. Microencapsulation of Probiotics

2.2.2.1. Preparation of *Saccharomyces boulardii* and *Alkalihalobacillus clausii*

The methodology for preparing microorganisms was adapted from the protocol developed by Du Le and Son Trinh (2018). The specific strain used in this study, *Saccharomyces boulardii* CNCM-I745, was sourced in lyophilized form from a reputable pharmacy (Biocodex, Reflor® 250 mg).

To revive the lyophilized *S. boulardii*, one 250 mg sachet of Reflor was resuspended in 100 mL of YPD medium (Peptone 2% (w/v), Yeast Extract 1% (w/v), D-Glucose 2% (w/v)) and incubated at 37°C for 24 hours. The following day, the revived *S. boulardii* was inoculated onto YPD agar media using a loop (Agar 2% (w/v), Peptone 2% (w/v), Yeast Extract 1% (w/v), D-Glucose 2% (w/v)) and incubated at 37°C for 2 days. A single colony was selected and inoculated into 100 mL of YPD medium, which was incubated at 37°C for 2 days.

As *Alkalihalobacillus clausii* (N/R, O/C, SIN, T), the commercial preparation Enterogermina® contains *Alkalihalobacillus clausii* spores were used (10^8 cfu/mL). The preperats were inoculated into TS medium (Tryptic Soy Broth, Merck) and incubated at 37°C for 24 hours for revival. On the subsequent day, the revived *A. clausii* was inoculated onto TSA media (Merck) using a loop and incubated at 37°C for 2 days. A single colony was selected and inoculated into 100 mL of TS medium, which was incubated at 37°C for 2 days.

To harvest the cells, the cultures were centrifuged at 5000 rpm for 15 minutes at 4°C, followed by washing with sterilized 0.85% saline solution. The viable cell density during the harvesting phase ranged from approximately 10^9 to 10^{10} CFU/mL. The

microbial suspensions were stored in saline solution until further use for the microencapsulation of probiotic cells.

2.2.2.2. Preparation of Lupine Protein Isolate-Trehalose-Xanthan Gum Complex

The xanthan gum (Metro-326711), trehalose (Sigma-T9531), and lupin protein isolate (LPI) emulsion were made using Çabuk & Harsa (2015)'s approach. In summary, distilled water was mixed with a magnetic stirrer for approximately three hours at room temperature, containing 4% w/v LPI, 1% w/v xanthan gum, and 2% w/v trehalose. Following the dissolving process, the mixture was denatured for fifteen minutes at 121 °C.

After that, the denatured solution was chilled and stored at 4 °C until probiotic cells were to be microencapsulated.

2.2.2.3. Preparation of Microcapsules with Emulsion Technique

For the microencapsulation of probiotics, the emulsion method, as outlined by Elvan, Baysal, and Harsa (2022), was used with necessary modifications. The initial water-in-oil emulsion was generated by emulsifying an internal aqueous phase composed of a protein-polysaccharide polymer blend (60%) containing microorganisms into an oil phase (40%) that includes 1% soy lecithin as an emulsifier.

Subsequently, the primary emulsion underwent homogenization using an Ultra Turrax homogenizer (Bandelin Sonopuls Hd 2070 homogenizer in Ultrasonic Technology) at 15000 rpm for 5 minutes. The emulsion was then added to a 100mM $CaCl_2$ solution, equal in volume to the emulsion, and subjected to additional homogenization for 2 minutes using the same homogenizer.

Following the formation of microcapsules, the resulting slurry was subjected to orbital shaking at 160 rpm for 30 minutes to facilitate the hardening of the microcapsules.

Subsequent to this step, the hardened microcapsules will be separated from the solution and oil phase through centrifugation at 1000 rpm for 1 hour.

2.2.2.4. Freeze Drying Microcapsules

The collected microcapsules underwent freezing at -20 °C and the microcapsules were freeze-dried with a Lablanco freeze dryer (Freezone 18, Kansas, USA) for 48 hours at - 55 °C under 0.050 mBar vacuum (Elvan et al., 2022). Lyophilized microcapsules then were stored at 4 °C for analysis.

2.2.3. Viable Counts of Microencapsulated Probiotics

The enumeration of microcapsules involved the utilization of the spread plates method on yeast extract peptone dextrose agar (YPDA) (Merck, Darmstadt, Germany) for *S. boulardii* and (TSA) (Merck, Darmstadt, Germany) for *A. clausii*. For each type of microcapsule, a suspension comprising 1 g of microcapsules was created in 9 mL of sterile pepton water and agitated on a magnetic stirrer for a duration of 30 minutes. Subsequent to this, serial dilutions were prepared, and the resultant mixtures were inoculated onto agar plates. Incubation of the plates was conducted at 37 °C for a period of 48 hours in accordance with the methodology outlined by Arslan et al. (2015). The bacterial counts were subsequently quantified and expressed as log10 colony-forming units per gram (cfu/g).

2.2.4. Microencapsulation Efficiency

The assessment of encapsulation efficiency involved an examination of the number of bacteria encapsulated within the microcapsules as described by Elvan, Baysal,

and Harsa (2022). For each type of microcapsule, one gram was introduced into 10 milliliters of phosphate buffer and vortexed for a duration of 5 minutes. Following the preparation of serial dilutions, culturing was performed on TSA medium for *A. clausii* and YPD medium for *S. boulardii*. The calculation of the encapsulation efficiency for the bacterial formulation was achieved using the subsequent formula (Equation 2.1.):

$$\text{Encapsulation efficiency (\%)} = \frac{N}{N_o} \text{ x } 100 \tag{2.1.}$$

In this equation, N represents the count of viable cells of *S.boulardii* and *A.clausii* following the microencapsulation process, and N_0 corresponds to the count of viable cells of *S.boulardii* and *A.clausii* prior to the microencapsulation process.

2.2.5. Evaluation of Microcapsule Stability and *In Vitro* Release

Free lyophilised microorganisms, microencapsulated microorganisms and microorganisms present in probiotic bars underwent exposure to simulated gastrointestinal conditions followed by a survival assessment. In the *in vitro* digestive tract, each of them will undergo transformation according to the INFOGEST protocol as described by Brodkorb et al. (2019). The oral phase solution for *in vitro* preparation comprised of 1.5 mM $CaCl_2$ and 75 U/mL amylase. To this, 1 g of probiotic bar, free lyophilised microorganisms and microencapsulated microorganisms sample was added and mixed with 2 mL of the oral phase solution in a flask. Subsequently, the flask was incubated at 37 °C for 2 minutes. After the incubation, a 1 mL sample was extracted. The final pH of the solution was adjusted to 7.0. For the *in vitro* preparation of the gastric solution, 0.15 mM $CaCl_2$, 2000 U/mL pepsin, and 60 U/mL lipase were used. The pH of the solution was adjusted to 3.0 by using 0.1 N HCl. Three millilitres of gastric solution was added to 3 grams of probiotic bar-oral phase mixture and incubated at 37 degree Celsius for 2 hours. At the end of the incubation period, 1 millilitre of samples were collected. To prepare the *in vitro* intestinal solution, a mixture of 10 millimoles of bile salt, 0.6 millimoles of $CaCl_2$, and 100 units/millilitre of pancreatin were used, and the pH of the solution was adjusted to 7 with 0.1 N sodium hydroxide. Then, 5 millilitres of

simulated gut solution was added to 5 grams of probiotic bar-gastric phase mixture and again incubated at 37 degree Celsius for 2 hours. At the end of the experiment, a 1 mL sample was collected and analyzed for the presence of *A. clausii* and *S. boulardii* during the simulated digestion test.

2.2.6. Microscopic Investigation of Microencapsulated Probiotics

The diameters and surface structures of the microcapsules are analysed by phase contrast microscopy (OLYMPUS-CX31) and scanning electron microscopy (SEM FEI QUANTA 250 FEG).

2.2.7. Preparation of Bar Formulation and Addition of Microencapsulated Probiotics

The formulation was meticulously prepared through the individual weighing of each ingredient, followed by manual mixing to create the final product. The constituents utilized include 120 grams of gluten-free oats, 200 grams of dates, 125 grams of peanut butter, 150 grams of dried figs, 100 grams of walnuts, 75 grams of flax seeds, 1 gram of cinnamon, and 1 gram of salt. The ingredients were procured from relevant retail or wholesale outlets. Peanut butter will function as both a binding agent and a carrier for the probiotics.

Upon combining all the ingredients, the resulting mixture was poured into a designated container. Since it is in a sticky form, it was pressed, compressed and brought together in the container. The mixture spread into a container meticulously cut into pieces measuring 5x3x2 (W*L*H) cm^3 square prismand weighing 10 grams each.

To produce 5 distinct types of bars, probiotic microorganisms were introduced into certain amounts of the specific microbial component and binding agent of the bars, which is the peanut butter. The following are exemplified variations of snack bars, each prepared with a specific microbial component:

PB C: Control bar samples (devoid of microorganisms).

PB F SB: Bar samples incorporating free *S. boulardii* microorganisms (non-microencapsulated).

PB ME SB: Bar samples incorporating microencapsulated *S. boulardii* microorganisms.

PB F BC: Bar samples incorporating free *B.clausii* microorganisms (non-microencapsulated).

PB ME BC: Bar samples incorporating microencapsulated *B.clausii* microorganisms.

2.2.8. Viable Counts of Microencapsulated Probiotics in Bar

Probiotic bars were stored in the refrigerator (± 4 °C). The enumeration was performed using the traditional spread plate method described by Grosso and Fávaro-Trindade (2004). Viability analyses of free lyophilised microorganisms, microencapsulated microorganisms and microorganisms present in probiotic bars were carried out on days 0, 15, 30, 60, and 90. In accordance with the Turkish Food Codex, food ought to possess no less than 1.0×10^6 KOB/g live probiotic microorganisms (TGKY, 2001). The shelf life shall thus be established whilst ensuring that the live microorganisms within the probiotic bars remain viable and are subject to monitoring throughout the storage process. It is imperative that the viability analysis does not indicate results lower than 1.0×10^6 KOB/g to meet the aforementioned requirement.

2.2.9. Moisture Content and Water Activity

The AOAC 934.06 was used to assess the probiotic bar's moisture content. To remove the moisture and stabilize the weight of the aluminum plates, they were placed in an oven set at 110°C for a whole night. Five grams of the homogenized material were placed on an aluminum dish (M0) that had been previously weighed. The sample and the dish were weighed prior to drying (M1). It was dried for six hours at 70°C under 100 mm-Hg in a vacuum oven (VO200, Memmert, Büchenbach, Germany). Following that, the

dish and hot sample were allowed to cool in a desiccator that included silica beads. As a last measurement, the dish and the dried sample were weighed (M2). The moisture content was calculated using the following formula (Equation 2.2.).

$$MC\ (\%) = \frac{M1 - M0}{M1 - M2}\ x\ 100 \qquad (2.2.)$$

A Hygrolab C1 water activity counter (Hygrolab C1, Rotronic, Bassersdorf, Switzerland) was used to measure the water activity of the probiotic bar samples (Dianawati, Mishra, & Shah, 2012).

2.2.10. Physicochemical Analysis

To ascertain the nutritional content of the probiotic bar, the following equation was used to calculate the carbohydrate content: % carbohydrate = 100 - (% moisture + % protein + % lipids + % ash). The Kjeldahl method (AOAC 2002) was employed to determine the protein content. The fat content was established by using the Soxhlet method as detailed by Manirakize et al. (2001), and the moisture content was calculated by subjecting the sample to oven drying at 105 °C until a constant weight has been achieved while following AOAC (2012) procedures.

2.2.11. Microbiological Quality Analyzes

Probiotic bars at a 1:10 ratio were suspended in peptone water using samples from each formulation. Following that, serial dilutions were made and used to evaluate the probiotic bars' survivability.

Using the spread plate technique, the amount of live cells in the product— *Saccharomyces boulardii* CNCM-I745 and *Alkalihalobacillus clausii* (N/R, O/C, SIN, T)—was quantified by cultivating them on yeast extract peptone dextrose agar (YPDA) (Merck, Darmstadt, Germany) and (TSA) (Merck, Darmstadt, Germany), respectively.

For 48 hours, the incubation procedure was conducted at 37°C in an aerobic environment. The weekly counts obtained were reported as log colony-forming units per gram (log CFU/g) using the Rossi et al. (2008) approach. The probiotic bars' microbiological safety was determined by counting yeast and mold and looking at *Escherichia coli*. VRB Agar (Violet Red Bile Agar, Merck, Germany) was used for the examination of *Escherichia coli*, and it was then incubated at 25°C for 48 hours. PDA (Potato Dextrose Agar, Oxoid, England) was used for the examination of yeast and mold, and it was incubated for 120 hours at 30°C.

2.2.12. Color Measurements

Color assessments of the probiotic bars were conducted using a Konica Minolta colorimeter, specifically the CR 410 model, manufactured by Konica Minolta in Tokyo, Japan described by Nkhata, (2020). The measurements were carried out in accordance with the CIE Lab color space system, which is characterized by L*, a*, and b* rectangular coordinates. In this system, L* represents luminance or lightness, a* signifies the red-green axis, and b* corresponds to the yellow-blue axis. Color measurements included the calculation of total color change (ΔE*), chroma (C*), and hue (h). The total color change (ΔE*) was calculated using the formula:

$$\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2} \qquad (2.3.)$$

where ΔL, Δa*, and Δb* represent changes in lightness, redness, and yellowness, respectively, after a specified period of time (month). Chroma (C*) was calculated using the formula:

$$C* = \sqrt{(a)^2 + (b)^2} \qquad (2.4.)$$

where a* and b* represent the a* value and b* value after a specified period of time (month). Hue (h) was calculated using the formula:

$$h = arctan \frac{b}{a} \qquad\qquad (2.5.)$$

where b* and a* represent the a* value and b* value, respectively. These calculations demonstrated that the total color change, chroma, and hue of the bars remained within acceptable ranges, ensuring the product's visual appeal over time.

2.2.13. Texture Properties

The textural characteristics of the probiotic bars were assessed using a texture analyser (TA.XT. plus, Stable micro systems, London, England) described by Kim et al., (2009).

Repeated analyses included four measurements using a quarter of the snack bar with dimensions of 5x3x2 (WLH) cm³, forming a square prism weighing 10 grams each for both assessments. A 75 mm diameter aluminum compression cylinder with a 60 degree angle 'V' groove was used for the texture profile examination. A cell load of fifty kilograms was delivered to seventy-five percent of the sample's initial thickness during two compressions. The specimen was positioned on a platform 20 cm from the plate, and the plate's speed was kept constant at 0.83 mm/sec.

For the shear test, a blade of 10 mm length and 1 mm thickness was used. The measurement was taken after the blade had completely cut through the probiotic bar, with a cell load of 50 kilograms at a knife distance of 20 cm and a cutting speed of 0.83 mm/sec. Texture analysis provided values for hardness, springiness, cohesiveness, gumminess, adhesiveness, chewiness and resilience.

Figure 6 describes Texture profile analysis (TPA), illustrating the force (g) applied over time (seconds) during test. The graph shows two peaks corresponding to two compression cycles, which provide insights into the texture characteristics.
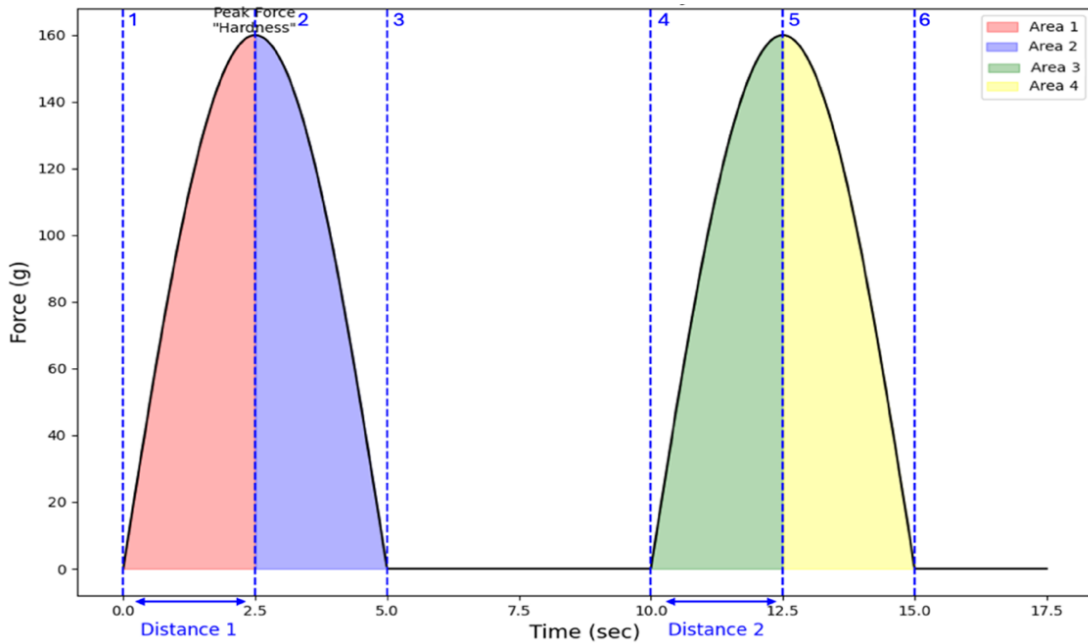
Figure 6: Texture profile analysis (TPA) graph

Table 2 ilustrates the parameters of Texture Profile Analysis (TPA) and how each parameter is calculated. These parameters provide a detailed analysis of the textural properties of the product and offer valuable insights for use in product development processes.

Table 2: Texture Profile Analysis Parameters and Measurements (Kim et al., 2009)

Parameter	Expression	Measurement
Hardness The peak force encountered during the initial phase of compression. While it is common for hardness to be observed at the maximum depth of compression, this is not always required for every product.	Maximum force of the first compression.	Peak Force at first compression
Cohesiveness The ability of the product to endure a second deformation compared to its resistance during the initial deformation.	The area of work during the second compression divided by the area of work during the first compression.	Area (3+4)/Area (1+2) optional (similar, not identical): Area 3/Area 2

(cont. on next page)

Table 2 (cont.)

Springness The extent to which a product recovers its shape after being deformed during the initial compression, once it has rested for the designated wait time between strokes. The springback is assessed during the down-stroke of the second compression. In some scenarios, a prolonged wait time may result in greater springback than what would occur under typical conditions being studied (for example, waiting 60 seconds between chews is not practical).	Springiness is often expressed as a ratio or percentage of the initial downstroke compression. It is typically measured by comparing the height detected during the second compression to the original compression distance.	Distance 2 / Distance 1
Gumminess Gumminess applies only to semi-solid products	Gumminess and chewiness are mutually exclusive properties, as a product cannot be both a semi-solid and a solid simultaneously.	Hardness x Cohesiveness
Chewiness Chewiness applies only to solid products	Gumminess * Distance 2 / Distance 1	Hardness x Cohesiveness x Springiness
Resilience Resilience refers to a product's ability to "recover its original height." This characteristic is assessed during the withdrawal phase of the first penetration, prior to the onset of the waiting period. Resilience can be evaluated with a single compression, provided that the withdrawal speed matches the compression speed.	Determined by dividing the energy expended during the upstroke of the first compression by the energy used during the downstroke of the same compression.	Area 2/Area 1

2.2.14. Sensory Analysis

The sensory panel for this study consisted of 30 untrained individuals. An acceptance test focusing on various sensory attributes such as appearance, flavour, colour, texture, taste and overall acceptability was conducted using a 5-point hedonic scale, where 1 indicates "not very much liked" and 5 indicates "very much liked" (Ihuoma et al., 2022). The sensory analysis took place one week after the production of the probiotic bars.During each evaluation session, panelists evaluated two probiotic bar formulations simultaneously. To maintain objectivity and avoid bias, each probiotic bar sample was assigned a unique 3-digit random number and presented to the panelists in a randomised

order. This approach ensured an unbiased and systematic evaluation of the sensory qualities of the probiotic bars.

2.2.15. Statistical Analysis

All experiments were meticulously conducted concurrently, and the presentation of results was accomplished with accompanying standard deviations. The subsequent data analysis was executed through the utilization of Minitab 18.0 software, developed by Minitab Inc. and headquartered in State College, PA, USA. To evaluate distinctions among the samples, statistical analysis involved the implementation of both a variance analysis (ANOVA) test and Tukey's test.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Viable Counts of Microencapsulated Probiotics

The study evaluated the survivability of probiotics *Saccharomyces cerevisiae* var. *boulardii* and *Alkalihalobacillus clausii* in different formulations, including free, microencapsulated-freeze dried, and microencapsulated-wet forms, over a duration of eight weeks at 4°C. The objective of these tests was to ascertain the efficacy of microencapsulation, particularly when combined with freeze drying, in preserving the viability of probiotic microorganisms, given the acknowledged significance of maintaining an adequate number of viable cells for the potential health benefits. It is crucial to note that the majority of health regulatory agencies recommend a minimum threshold of 6.0 log CFU/g for probiotic effectiveness. Table 3 shows cell survivals of *Saccharomyces cerevisiae* var. *boulardii* and *Alkalihalobacillus clausii* in different forms for 8 weeks at 4 °C and Figure 7 shows the stability of free and microencapsulated forms of *Saccharomyces cerevisiae* var. *boulardii* and *Alkalihalobacillus clausii* cells.

Following eight weeks of storage, the viability of *S. boulardii* decreased by 2 log in the free form and by 0.5 log in the microencapsulated-freeze dried form. In contrast, a 1.4 log decrease was observed in the microencapsulated-wet form over the same period. These findings demonstrate that freeze drying may offer a more effective approach to viability preservation than the free and microencapsulated-wet forms.

In the case of *B. clausii*, the free form displayed a viability loss of 2 log after 8 weeks, whereas the microencapsulated-freeze-dried form experienced a notably smaller decline of 0.4 log. The microencapsulated-wet form, however, exhibited a reduction of 1.7 log, indicating a less efficient preservation of viability compared to the freeze-dried counterpart.

Table 3: Cell survival of free, microencapsulated-freeze dried and microencapsulated-wet forms of *Saccharomyces cerevisiae* var. *boulardii* and *Alkalihalobacillus clausii* for 8 weeks at 4 °C.

Time \\ Probiotic	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
FREE SB	9.4246 ± 0.0917^{aA}	9.3205 ± 0.306^{aA}	9.0915 ± 0.221^{aAB}	8.9370 ± 0.0727^{aABC}	8.4414 ± 0.187^{aCD}	8.1312 ± 0.197^{aDE}	7.9451 ± 0.1717^{aDE}	7.5124 ± 0.399^{abF}
ME FD SB	8.5391 ± 0.1625^{bAB}	9.0483 ± 0.639^{abA}	8.5026 ± 0.1254^{abAB}	8.5720 ± 0.0651^{abAB}	8.2908 ± 0.187^{abAB}	8.2535 ± 0.216^{abAB}	8.0942 ± 0.243^{aB}	8.0681 ± 0.332^{aB}
ME W SB	8.8817 ± 0.0915^{abA}	8.5896 ± 0.1232^{abcA}	$8,4348 \pm 0.316^{abcA}$	8.4480 ± 0.0760^{bA}	8.2882 ± 0.427^{abAB}	7.7481 ± 0.1645^{bcBC}	7.5290 ± 0.1052^{abCD}	7.4032 ± 0.245^{bcD}
FREE BC	8.5069 ± 0.412^{bA}	8.4061 ± 0.288^{abcA}	8.1884 ± 0.438^{bcA}	7.9934 ± 0.313^{cAB}	$7.7091 \pm 0.0172^{bcABC}$	$7.2282 \pm 0.1634^{cdBCD}$	6.9264 ± 0.440^{bCD}	6.4129 ± 0.1622^{cD}
ME FD BC	8.0487 ± 0.313^{bA}	7.9531 ± 0.575^{cA}	7.8858 ± 0.1637^{bcA}	7.8231 ± 0.1594^{cA}	7.7983 ± 0.266^{bcA}	7.8205 ± 0.252^{bcA}	7.5463 ± 0.390^{abA}	7.6433 ± 0.253^{abA}
ME W BC	8.3249 ± 0.547^{bA}	7.9648 ± 0.113^{bcAB}	7.7402 ± 0.134^{cABC}	7.6378 ± 0.0754^{cABC}	7.4390 ± 0.397^{cABCD}	7.0823 ± 0.305^{dBCD}	6.8326 ± 0.494^{bCD}	6.5180 ± 0.205^{cD}

Notes: Results are shown as means ± standard deviation. Columns with different lowercase letters differ statistically ($p \leq 0.05$). Rows with different uppercase letters differ statistically ($p \leq 0.05$).

The results presented herein demonstrate the crucial role of freeze drying in extending the viability and shelf life of probiotic bacteria. Furthermore, the results indicate that moist microencapsulation is not an optimal method for maintaining viable counts over extended storage periods. In order to ensure the potential health advantages and efficacy of probiotics, it is essential that formulations with viable counts higher than the minimal criterion of 6.0 log CFU/g are included.
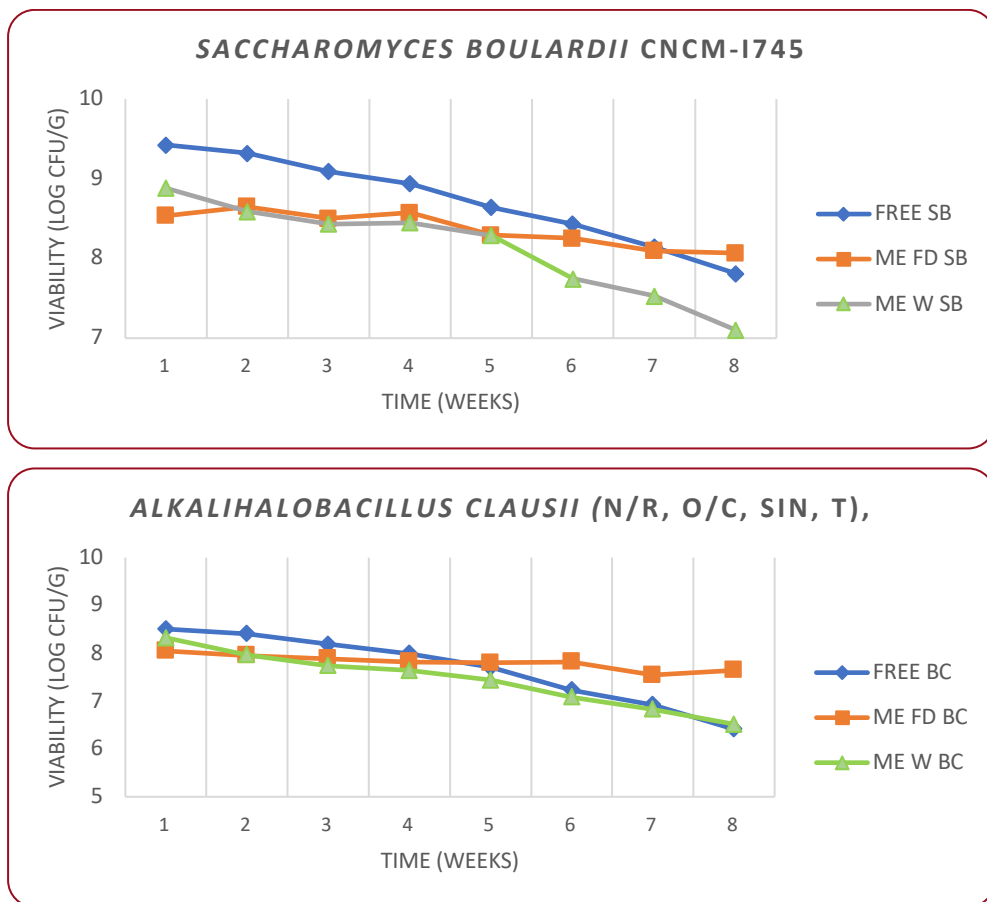
Figure 7: Viability of *Saccharomyces boulardii* CNCM-I745 and *Alkalihalobacillus clausii* (N/R, O/C, SIN, T) during 8 weeks of storage at 4 ∘C

In the study conducted by Niamah et al., 2018, the viability of *Saccharomyces boulardii* in ice cream mixtures was investigated, with a focus on the effects of encapsulation on cell survival during freezing and frozen storage. It was observed that even with a high percentage (4%) of inoculum, *S. boulardii* exhibited robust growth in

ice cream mixtures, regardless of whether the cells were in free form or encapsulated. However, encapsulation led to a decrease in viable cell count from 8.55 to 5.25 log CFU/g. Freezing was found to have a significant detrimental effect on the viability of probiotic cells, resulting in a 3.3 log cycle decrease in the count of free cells compared to encapsulated cells, which experienced decreases of 2.51 and 1.51 log cycles in Treatment II and Treatment III, respectively. Encapsulation, particularly with alginate microbeads, was shown to effectively protect probiotic cells against freezing injuries during both freezing and frozen storage, as evidenced by minimal decreases in viable cell count over time. These findings align with previous studies by Ahmadi et al. (2012), Homayouni et al. (2008), Akin et al. (2007), and Heydari et al. (2012), which have also demonstrated the protective effects of encapsulation on probiotic viability in frozen dairy products.

The emulsion approach was utilized to accomplish probiotic microencapsulation under sterile circumstances in the study carried out by Halimi et al., 2022. For 28 days, at 7-day intervals (0, 7, 14, 21, 28 days) and temperatures (4, 20, 35 °C), the number of probiotics and physical analysis (pH, stability, and organoleptic characteristics) of 120 heated Doogh samples containing free and encapsulated *L. acidophilus* and *S. boulardii* probiotics were carried out. Therefore, probiotic survival declined steadily during the course of storage at all temperatures, with the exception of the encapsulated forms of *L. acidophilus* at 4 and 20 °C and the relatively constant free forms of *S. boulardii* at 4 °C ($P < 0.05$). During the final day of storage, at 20 and 35 ºC, the treatments that included free *S. boulardii* showed the least significant pH change.

In conclusion, the research findings indicate that freeze drying is the optimal method for microencapsulating probiotic microorganisms, as it enhances their viability and stability. This will facilitate the incorporation of microorganisms into functional foods and dietary supplements with longer shelf lives and higher levels of effectiveness.

3.2. Microencapsulation Efficiency

Table 4 presents the survival rates of *Saccharomyces cerevisiae* var. *boulardii* and *Alkalihalobacillus clausii* after microencapsulation and storage at 4°C for 8 weeks. For *S. boulardii*, the initial cell viability was 8.54 log CFU/g, which decreased to 8.07

log CFU/g by the end of the 8-week period. The cell survivability for *S. boulardii* started at 90.61% in the first week and dropped to 85.61 % in the eighth week. These results indicate that while microencapsulation effectively maintained the viability of *S. boulardii*, a statistically significant decline was observed, particularly in weeks 7 and 8, as denoted by different superscripts (A and B) within the same column (P<0.05).

For *B. clausii*, the initial cell viability was 8.05 log CFU/g, which reduced to 7.64 log CFU/g after 8 weeks. The cell survivability began at 94.62 % and decreased to 89.85 % over the same period. The survivability rates for *B. clausii* were generally higher and exhibited less reduction over time compared to *S. boulardii*. Notably, there were no significant differences in cell viability within the same column across different weeks, indicating consistent survival rates throughout the storage period.

Table 4: Survival of *Saccharomyces cerevisiae* var. *boulardii* and *Alkalihalobacillus clausii* after microencapsulation for 8 weeks at 4 °C.

Time (Week)	Cell viability of *S. boulardii* after microencapsulation (log CFU/g)	Cell Survivability of *S .boulardii* (%)	Cell viability of *B. clausii* after microencapsulation (log CFU/g)	Cell Survivability of *B .clausii* (%)
1	8.5391 ± 0.1625^{AB}	90.6041	8.0487 ± 0.313^{A}	94.6140
2	9.0483 ± 0.639^{A}	96.0065	7.9531 ± 0.575^{A}	93.4900
3	8.5026 ± 0.1254^{AB}	90.2170	7.8858 ± 0.1637^{A}	92.6995
4	8.5720 ± 0.0651^{AB}	90.9529	7.8231 ± 0.1594^{A}	91.9618
5	8.2908 ± 0.187^{AB}	87.9690	7.7983 ± 0.266^{A}	91.6707
6	8.2535 ± 0.216^{AB}	87.5736	7.8205 ± 0.252^{A}	91.9318
7	8.0942 ± 0.243^{B}	85.8831	7.5463 ± 0.390^{A}	88.7083
8	8.0681 ± 0.332^{B}	85.6068	7.6433 ± 0.253^{A}	89.8487

Notes: Results are shown as means ± standard deviation. Columns with different uppercase letters differ statistically (p≤0.05).

Overall, the microencapsulation technique demonstrated a substantial protective effect on the probiotics, with *B. clausii* showing a more pronounced stability compared to *S. boulardii*. These findings underscore the efficacy of microencapsulation in preserving probiotic viability during storage, although some degree of viability loss is inevitable over extended periods. The statistically significant reduction in the viability of *S. boulardii* in the latter weeks suggests that further optimization of the microencapsulation process may be necessary to enhance the long-term stability of this probiotic strain.

In a study by Guowei et al. (2019), composite cryoprotectants were optimised in order to enhance the survival rate of *Saccharomyces cerevisiae* var. *boulardii* during freeze-drying. The optimised formulation demonstrated a high survival rate of 64.22% and promising results in an accelerated storage test. This study serves to illustrate the potential application of optimised cryoprotectants in various industries. The findings of this study align with the research objectives of this laboratory, namely to assess the viability of microencapsulated probiotics, particularly in freeze-dried forms, for the purpose of extending shelf-life.

Six distinct wall materials (gelatin, whey protein concentrate, modified starch, maltodextrin, pea protein isolate, and gum Arabic) were used to microencapsulate *S. boulardii* in a research by Arslan et al. (2015). The samples were then spray dried at two different input temperatures (80 °C and 125 °C). With whey protein concentrate (92%) and gum Arabic (90%), the maximum product yield was obtained. At the lower drying temperature of 80°C, the probiotic count rose with survival rates of 84,69% and higher, but it remained stable across wall materials.

3.3. Evaluation of Microcapsule Stability and *In Vitro* Release

In the simulated gastric fluid at pH 2.0, all strains exhibited significantly lower viability compared to other pH levels. The viability of all strains decreased significantly during the 180-minute simulated gastric transit. The reductions were 5.1 log, 2.2 log, and 3.5 log for *S. boulardii*, and 3.2 log, 1.5 log, and 2.4 log for *B. clausii*, respectively. It is clear that the pre-existing capsules of *B. clausii* make it more resilient to acidic conditions compared to *S. boulardii*, which is why it exhibits comparatively lower reductions in

viability. At pH 3.0 of the simulated gastric fluid, reductions in viability over 180 minutes were observed to be 1.85 log, 0.6 log, and 1.3 log for *S. boulardii*, and 2 log, 1.2 log, and 1.5 log for *B. clausii*, respectively. In the pH 4.0 simulated gastric fluid conditions, all strains maintained the same level of viability throughout the 180-minute transit. Furthermore, the presence or absence of bile salts did not significantly impact the viability of any tested strains during the 180-minute simulated small intestinal transit. Both *S. boulardii* and *B. clausii*, whether free or microencapsulated (freeze-dried or wet), demonstrated nearly identical viability in the presence and absence of 0.3% bile salts. This indicates robust resistance of the strains to bile salts under the experimental conditions.

Overall, the microencapsulation, particularly the freeze-dried form, provided a substantial protective effect, enhancing the survival rates of both *S. boulardii* and *B. clausii* under simulated gastrointestinal conditions. These findings highlight the potential of microencapsulation in improving the stability and efficacy of probiotic formulations under harsh gastrointestinal environments. Figure 8 illustrates the viability of free, microencapsulated-freeze dried, and microencapsulated-wet forms of *Saccharomyces cerevisiae* var. *boulardii* and *Alkalihalobacillus clausii* both before digestion and at the end of the simulated *in vitro* digestion.

In a study by Le, H. D., & Trinh, K. S. (2018), three probiotic species, *Lactobacillus acidophilus, Bacillus clausii,* and *Saccharomyces boulardii*, were encapsulated using a hydrogel encapsulation technique. Sodium alginate and gelatin served as the first and second coating agents, respectively. Both free and encapsulated probiotic cells were then inoculated in gastrointestinal media for 120 minutes to assess the protective efficacy of the encapsulation layers. The results demonstrated that free bacteria were completely inactivated after 90 minutes in low pH conditions, whereas those encapsulated with sodium alginate exhibited a higher viable cell density. Double-layer encapsulation significantly enhanced cell survival, maintaining the viable cell density of all three probiotics for the full 120 minutes. Free yeast demonstrated superior acid tolerance compared to bacteria, with over 3 log survival after 120 minutes at pH 2.0. Encapsulated yeast exhibited a stable viable cell density throughout the period. The tolerance of the three species under intestinal conditions followed a similar pattern to that observed under gastric conditions.
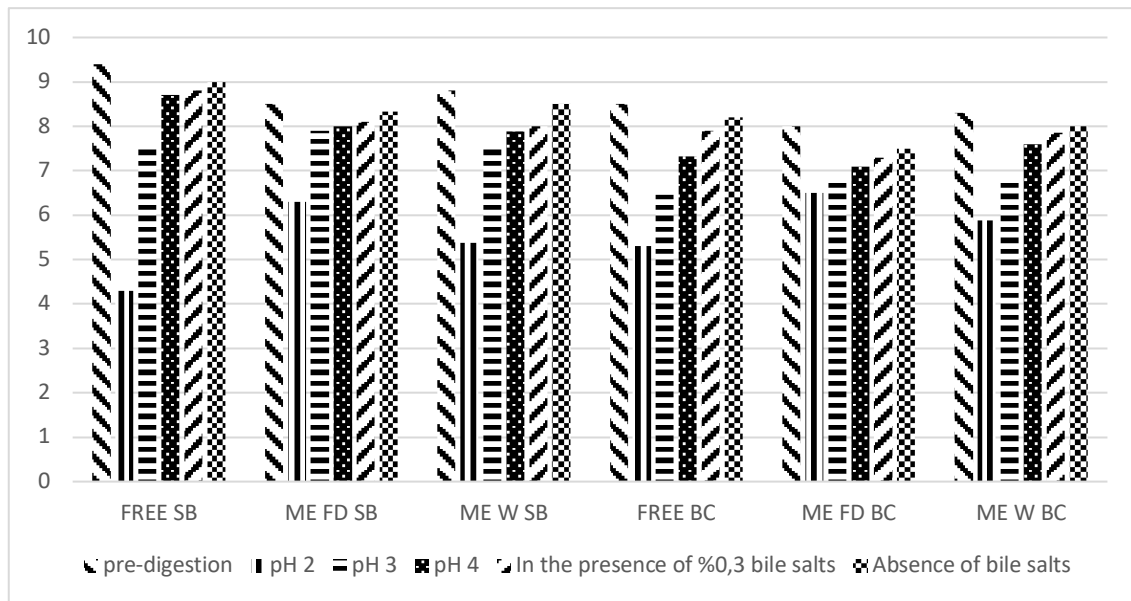
Figure 8: Viability of free, microencapsulated-freeze dried and microencapsulated-wet forms *Saccharomyces cerevisiae* var. *boulardii* and *Alkalihalobacillus clausii* pre-digestion and at the end of the simulated *in vitro* digestion

In a previously mentioned study conducted by Arslan et al. (2015), it was also founded that, *S. boulardii* microcapsules produced at 125 °C exhibited greater resistance to gastric solution, with counts of 5.89-6.06 log cfu/g, compared to those dried at 80 °C. In simulated gastric tests, gum Arabic was the most effective wall material, followed by gelatin and pea protein, maintaining probiotic levels of 6.06-6.27 log cfu/g. Survivability decreased with increased exposure time to the gastric solution. These findings are consistent with previous studies on various *Lactobacillus* and *Bifidobacterium* species, which have demonstrated the influence of drying conditions and wall materials on probiotic viability during spray drying.

In a study conducted by Ersoy et al., (2023), the aim was to produce cake with probiotic properties using *B. clausii* spores. The baking processes included normal conditions, microwave, and steam-assisted methods. The viability of the probiotic spores in the cakes was assessed at the end of baking, under *in vitro* digestion conditions, and during storage. After baking, the probiotic spore content of the cake samples ranged from 5.74 to 5.88 log cfu/g, with approximately 79% viability preserved. Following gastric and intestinal digestion, the cake samples were found to contain *B. clausii* at levels of 5.89-

6.06 log cfu/g and 6.06-6.27 log cfu/g, respectively, indicating that the probiotic spores were highly resistant to gastrointestinal conditions. Storage tests showed that bacterial spores remained quite stable, maintaining their initial levels in the samples stored at refrigerator temperature. Higher bacterial spore viability was detected in the samples stored at refrigerator temperature.

3.4. Microscopic Investigation Of Microencapsulated Probiotics

The SEM images provided offer a detailed examination of the microencapsulation structures of two probiotic microorganisms: *Saccharomyces boulardii* (Figure 9) and *Bacillus clausii* (Figure 10). Both microorganisms were encapsulated using the same coating materials and microencapsulation technique.

Figures 9 depict the microencapsulated *S. boulardii*. The images reveal a consistent, well-defined encapsulation matrix surrounding the probiotic cells. The encapsulation appears to form a protective barrier, which is essential for preserving the viability of the yeast cells during storage and upon ingestion. The surface morphology shows a rough and irregular texture, indicating the complex nature of the encapsulating material, which could be a combination of biopolymers designed to enhance the stability and controlled release of the probiotics.
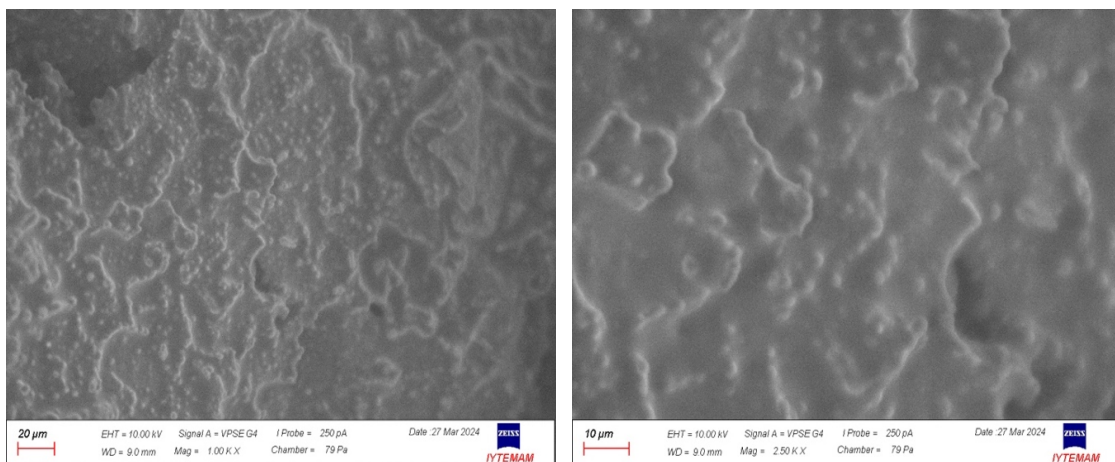

Figure 9: SEM images of microencapsulated *Saccharomyces boulardii*

Figure 10 show the microencapsulated *B. clausii*. The encapsulation matrix is also clearly visible, providing a similar protective barrier around the bacterial cells. Interestingly, despite using the same encapsulating materials and techniques, the surface morphology of *B. clausii* appears smoother and more homogenous compared to *S. boulardii*. This variation in surface texture might be attributed to the intrinsic structural differences between the yeast and bacterial cells, which could influence the interaction with the encapsulating materials.
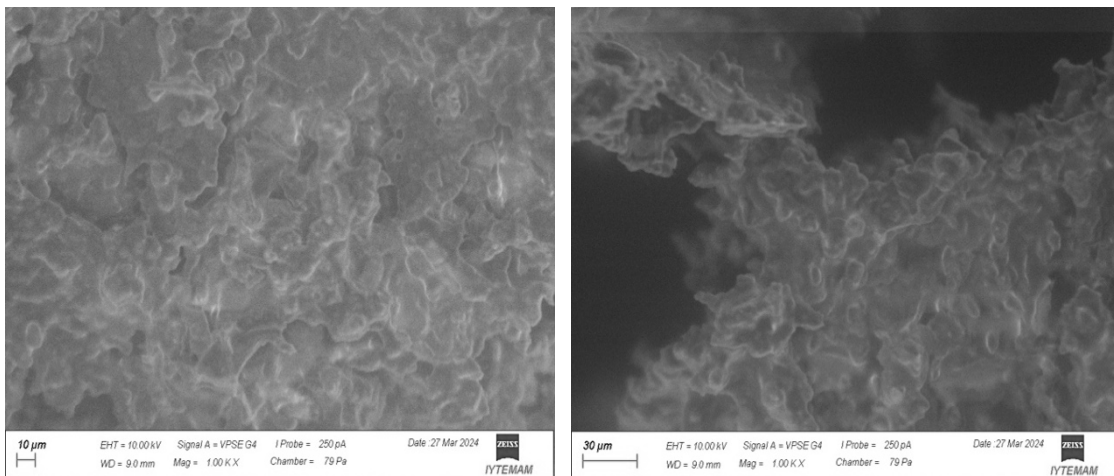
Figure 10: SEM images of microencapsulated *Bacillus clausii*

Overall, the SEM images underscore the importance of microencapsulation in maintaining the structural integrity and functionality of probiotic microorganisms. The distinct surface morphologies observed, despite using identical encapsulation processes, highlight the need to consider the specific characteristics of each probiotic species to optimize their stability and efficacy.

The phase contrast microscopy images provided illustrate the structural differences between free form and microencapsulated *Bacillus clausii* and *Saccharomyces boulardii* using the same encapsulation materials and technique.

Figure 11 a) shows *B. clausii* in its free form. The individual bacterial cells appear rod-shaped and are dispersed throughout the medium, displaying typical bacillary
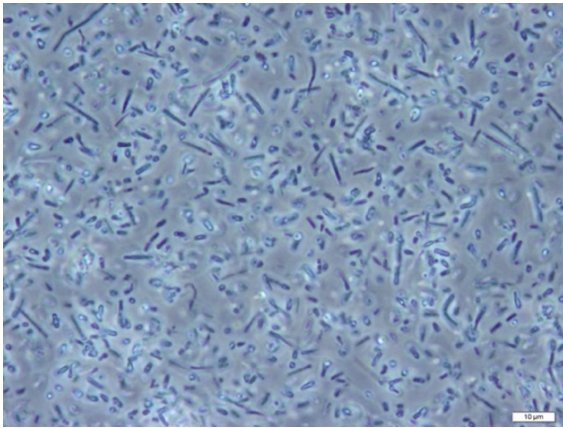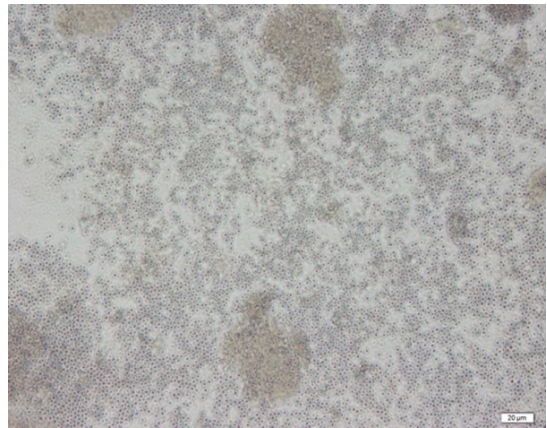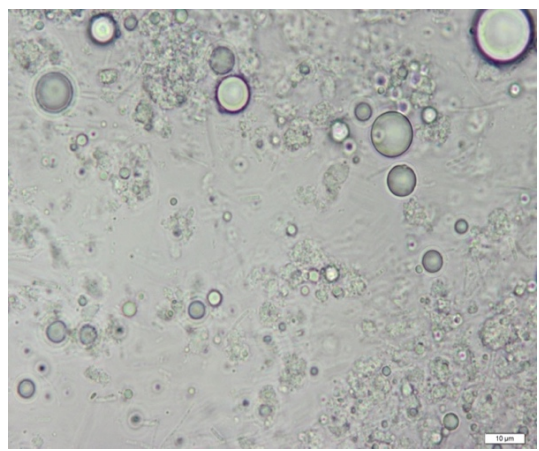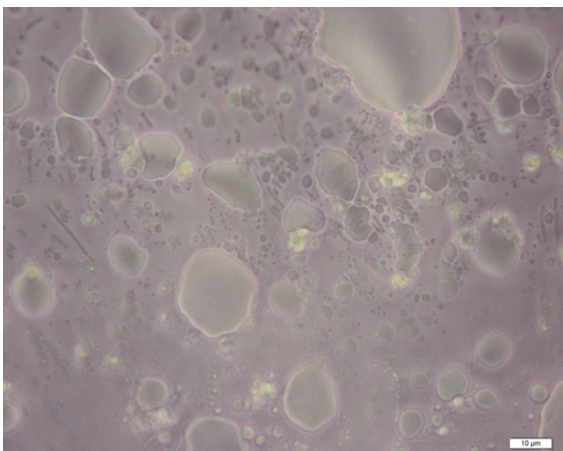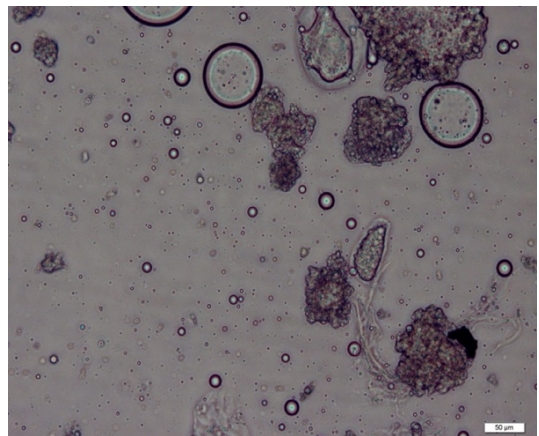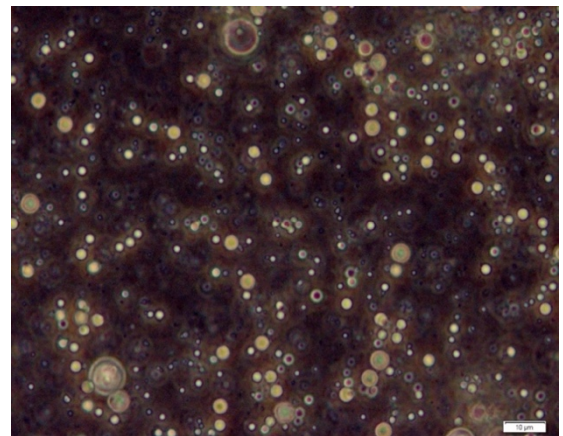
Figure 11: Phase Contrast images of a) Free *Bacillus clausii* b) Free *Saccharomyces boulardii* c) Microencapsulated *Bacillus clausii* d) Microencapsulated *Saccharomyces boulardii*

morphology. The clear visibility of each cell indicates an unprotected state with cells freely interacting with the surrounding environment. c) display *B. clausii* after microencapsulation. The encapsulation matrix forms distinct spherical structures around the bacterial cells. These microcapsules vary in size, suggesting heterogeneous encapsulation efficiency. The spherical shapes are well-defined and encapsulate multiple cells within each capsule, providing a clustered arrangement that contrasts with the dispersed nature of the free form.

Figure 11 b) shows *S. boulardii* in its free form. The yeast cells exhibit typical round or oval shapes, characteristic of yeast morphology. They are uniformly distributed across the field of view, with each cell easily identifiable and unprotected. D) depict *S. boulardii* after microencapsulation. Similar to *B. clausii*, the yeast cells are encapsulated within spherical structures. The microcapsules display a range of sizes, indicating a degree of variability in encapsulation. The encapsulated yeast cells are grouped together within each spherical structure, in contrast to the evenly dispersed free form cells.

In both microorganisms, the encapsulated forms show a clear distinction in structural organization compared to their free form states. The encapsulation process results in the formation of well-defined spherical microcapsules, which contain and protect multiple cells. This transformation from individual, dispersed cells to grouped, encapsulated cells is evident in the phase contrast images, highlighting the successful application of the encapsulation technique.

3.5. Viable Counts Of Microencapsulated Probiotics In Bar

The viability of free and microencapsulated *Saccharomyces boulardii* and *Bacillus clausii* strains was monitored over 0, 30, 60, and 90 days to evaluate their stability in a probiotic bar formulation stored at 4°C. The results are summarized in the Table 5.

The free form of *S. boulardii* started with an initial cell count of 8.52. By day 30, the viability dropped to 7.92, showing a statistically significant decrease (p≤0.05). This decline continued over the next two months, with cell counts reducing to 6.24 by day 60 and further plummeting to 4.84 by day 90. This represents a total log reduction of

approximately 3.5 logs over the 90-day period, highlighting the vulnerability of the free form cells to environmental stressors during storage. In contrast, the microencapsulated *S. boulardii* exhibited much greater stability. Starting at 8.09 on day 0, the cell count showed a slight decrease to 7.79 by day 30, with minimal reduction observed. By day 60, the count was 7.64, and by day 90, it remained relatively high at 7.42. This represents a total log reduction of about 0.65 logs, maintaining cell counts much higher than the free form over the 90-day period.

Table 5: Cell survival of free and microencapsulated *Saccharomyces cerevisiae* var. *boulardii* and *Alkalihalobacillus clausii* in probiotic bar formulation for 90 days at 4 °C.

Days \ Bar Type	0	30	60	90
PB F SB	8.5188 ± 0.1325^{abA}	7.9206 ± 0.281^{aB}	6.2355 ± 0.272^{bC}	4.8435 ± 0.368^{bD}
PB ME SB	8.0879 ± 0.0464^{bA}	7.7962 ± 0.1315^{aAB}	7.6392 ± 0.439^{aAB}	7.4231 ± 0.256^{aB}
PB F BC	9.0622 ± 0.6371^{aA}	8.2700 ± 0.7000^{aB}	6.5634 ± 0.343^{bC}	5.4206 ± 0.544^{bD}
PB ME BC	8.5837 ± 0.0559^{abA}	8.3552 ± 0.218^{aA}	8.2375 ± 0.267^{aA}	8.0781 ± 0.1298^{aA}

Notes: Results are shown as means ± standard deviation. Columns with different lowercase letters differ statistically (p≤0.05). Rows with different uppercase letters differ statistically (p≤0.05).

For *B. clausii*, the free form began with an initial count of 9.06. By day 30, the viability reduced to 8.27. This trend continued with a cell count of 6.5634 on day 60 and 5.4206 by day 90. Similar to *S. boulardii*, the free form of *B. clausii* represents a total log reduction of approximately 3.5 logs, similar to *S. boulardii*, emphasizing the need for protective measures to enhance stability. The microencapsulated *B. clausii* started with a cell count of 8.5837 on day 0. The viability remained high at 8.3552 by day 30, showing

minimal decrease. By day 60, the count was 8.2375, and by day 90, it was 8.0781. This represents a total log reduction of about 0.5 logs and indicates that microencapsulation provides substantial protection for *B. clausii*, significantly reducing the rate of viability loss compared to the free form.
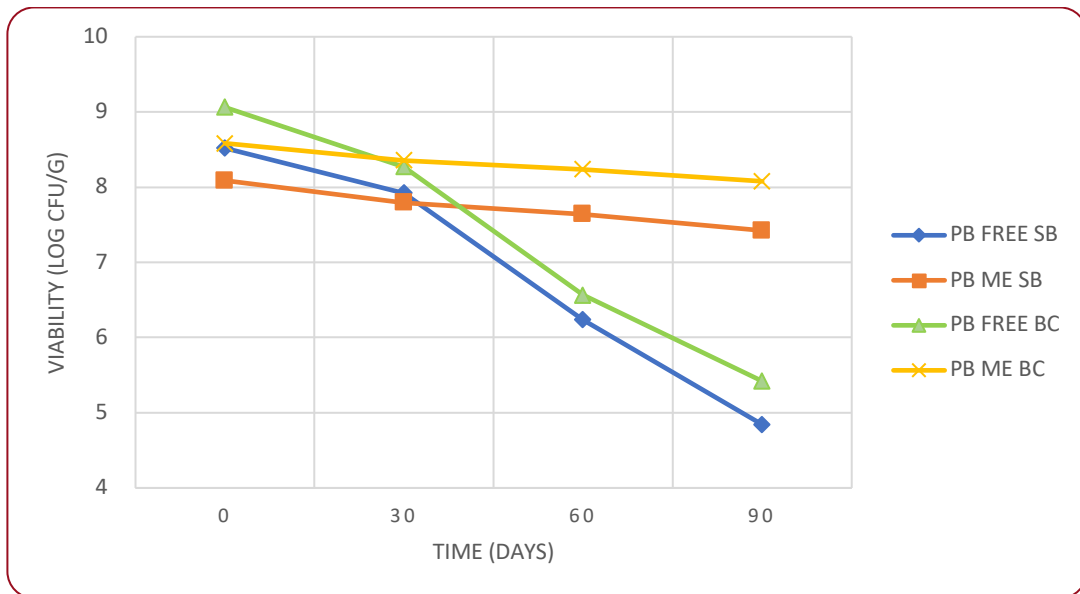
Figure 12: Viability of *S. boulardii* & *B.clausii* in probiotic bars during 90 days of storage at 4 °C.

Overall, the results demonstrate that microencapsulation significantly enhances the stability and viability of both *S. boulardii* and *B. clausii* over a 90-day storage period. The microencapsulated probiotics exhibit much lower log reductions in cell viability compared to their free form counterparts, underscoring the effectiveness of the encapsulation technique in preserving cell viability during storage as seen in Figure 12. This preservation is crucial for maintaining the functional efficacy of probiotic products over their shelf life.

The greater stability of microencapsulated forms in the bar formulation can be attributed to several key factors related to the encapsulation process and the nature of the bar matrix. Microencapsulation provides a protective barrier that shields the probiotics from environmental stressors, such as oxygen, moisture, and temperature fluctuations, which are common in food matrices. This encapsulating material forms a physical shell

around the probiotic cells, effectively isolating them from adverse conditions that would otherwise lead to cell damage and death.

In the context of a bar formulation, the matrix itself, often containing fats, can also play a significant role in preserving the viability of the probiotics. The lipid content in the bar can create a hydrophobic environment that further protects the encapsulated cells from moisture and oxygen, enhancing their stability. This fatty environment acts as an additional layer of protection, reducing the impact of environmental stressors and contributing to the prolonged viability of the probiotics.

However, the free forms of *Saccharomyces boulardii* and *Bacillus clausii* do not benefit from such protection. Without the encapsulating barrier, these cells are directly exposed to the bar's components and the surrounding environment. In this unprotected state, the probiotics can come into direct contact with the bar's nutrient sources. This exposure can stimulate metabolic activity, leading the cells to consume available resources and potentially enter a growth phase. While this might seem beneficial initially, it can hasten the onset of the stationary and death phases due to nutrient depletion and accumulation of metabolic byproducts, resulting in a significant loss of viability over time.

Moreover, the controlled release mechanism provided by encapsulation ensures that the probiotics are not prematurely activated or consumed within the bar. This isolation from direct nutrient sources helps maintain the cells in a dormant but viable state, ready to be activated upon consumption when they reach the gastrointestinal tract. In contrast, the free cells lack this regulation and are prone to rapid metabolic shifts, which can lead to quicker viability losses.

3.6. Microbiological Quality Analyses

The microbiological quality analyses were conducted over a 90-day period. Samples from each formulation were suspended in peptone water, and serial dilutions were prepared to assess the viability of probiotic bars. Quantification of live cells of *Saccharomyces boulardii* CNCM-I745 and *Alkalihalobacillus clausii* was performed on YPDA and TSA media, respectively, under aerobic conditions at 37°C for 48 hours. As

shown in Table 5, the probiotic bars maintained a high viability of these probiotic strains throughout the storage period, with results expressed as log CFU/g.

For safety assessments, *Escherichia coli* counts were performed using VRB Agar, incubated at 25°C for 48 hours. Additionally, yeast and mold counts were analyzed using PDA, incubated at 30°C for 120 hours. According to the Turkish Food Codex, the acceptable limit for yeast and mold in food products is below 10^4 CFU/g. The analysis showed no detectable levels of *Escherichia coli*, and yeast and mold counts were well within the acceptable limits, ensuring the microbiological safety of the probiotic bars throughout the 90-day storage period. These results confirm that the probiotic bars are both safe and of high microbiological quality, adhering to the standards set by the Turkish Food Codex.

Spim et al. (2021) conducted a study on the development and evaluation of food bars containing *Lentinula edodes* (shiitake). They found that the sweet bar variant (SwB1) maintained its nutritional quality and exhibited no microbial growth over a 180-day period, meeting the standards set by the National Health Surveillance Agency (ANVISA).

Similar to the findings of Ibrahim et al. (2021), who incorporated date fruit from the Kingdom of Saudi Arabia into snack bars, our study has demonstrated that date paste is an effective functional ingredient in probiotic bars. Their research showed that microbial analyses over a 12-day period revealed no pathogenic bacteria or visible mold, ensuring the product's safety. Likewise, our 90-day microbiological analysis confirmed no detectable *Escherichia coli* and acceptable levels of yeast and mold, adhering to Turkish Food Codex standards. These results underscore the microbiological safety and potential of date paste in functional food products.

3.7. Moisture Content

Using two replications, the moisture content of the probiotic bars was assessed at days 0, 30, 60, and 90 of each month's observation period. Table 6 displays the measured values. The Control Bar exhibited relatively stable moisture content, starting at 10.44% and experiencing slight fluctuations before stabilizing at 9.98% by day 90. This minimal change suggests that the Control Bar maintains its moisture well over time, which is

consistent with general observations in food stability studies where control samples often serve as benchmarks for stability (Viet et al., 2021).

Table 6: Results of probiotic bar's moisture content (%)

Days / Bar Type	0	30	60	90
PB C	10.4384 ± 0.027	10.4987 ± 0.854	9.9822 ± 0.482	9.7321 ± 0.692
PB F SB	11.6090 ± 0.478	10.3921 ± 0.023	10.4987 ± 0.729	9.8795 ± 0.382
PB ME SB	10.6166 ± 1.502	10.4623 ± 0.763	9.6399 ± 0.156	9.6543 ± 0.185
PB F BC	9.8529 ± 0.986	9.6753 ± 0.329	9.2685 ± 0.238	9.3245 ± 0.127
PB ME BC	10.8268 ± 0.464	10.2748 ±0.187	9.7627 ± 0.647	9.7456 ± 0.453

The FREE SB bar, which began with a moisture content of 9.98%, showed a notable increase to 10.39% by day 30 and further to 10.50 % by day 60, before dropping to 9.88% by day 90. This pattern indicates that while FREE SB initially absorbs moisture, it loses some of it towards the end of the storage period, yet still retains a relatively high moisture level. Such behavior is common in food products where initial moisture absorption can occur due to interactions with packaging or environmental conditions (Gaikwad et al. 2019).

In contrast, the ME SB bar, which started with the highest initial moisture content of 10,61%, saw a decrease to 10.46% by day 30 and a more significant drop to 9.64% by day 60. However, by day 90, its moisture content slightly recovered to 9.65%, indicating some stabilization. This trend suggests that while ME SB initially loses moisture, it achieves some balance towards the end of the storage period. This could be attributed to the protective effects of microencapsulation, which helps in retaining moisture better than free forms (Bakry et al., 2016).

The FREE BC bar exhibited a consistent decrease in moisture content, starting at 9.85% and dropping to 9.68% by day 30, then further to 9.27% by day 60, before slightly increasing to 9.32% by day 90. This consistent loss, with a minor recovery, highlights the difficulty of maintaining moisture in the FREE BC form over time. Such patterns are often seen in free probiotic forms, where the lack of protective encapsulation can lead to greater moisture loss (Šipailienė & Petraitytė, 2018). Finally, the ME BC bar started with the highest initial moisture content among all bars at 10.83%. It showed a decrease to 10.27% by day 30 and further to 9.76% by day 60, ending at 9.75% by day 90. Despite the reductions, ME BC consistently retained higher moisture content compared to FREE BC, suggesting that microencapsulation significantly aids in preserving moisture throughout the shelf life. This is in line with the general understanding that microencapsulation can enhance moisture retention and stability in food products (Hoyos-Leyva et al., 2018).

Overall, the microencapsulated bars (ME SB and ME BC) demonstrated better moisture retention compared to their free counterparts, particularly evident in the ME BC bar, which maintained the highest moisture levels consistently. These observations suggest that microencapsulation is effective in enhancing the moisture stability of probiotic bars over extended storage periods. This supports the broader findings in food technology research that encapsulation techniques can significantly improve the stability and longevity of bioactive compounds in various food matrices (Yan et al., 2022; Nami et al., 2020; Ye et al., 2018).

3.8. Water Activity

The water activity (aw) values for different types of bars were measured at 0, 30, 60, and 90 days to assess their stability over time. The measured values are shown in Table 7. The control bar showed a gradual decrease in water activity from 0.656 ± 0.004 at day 0 to 0.585 ± 0.003 at day 60, followed by a slight increase to 0.592 ± 0.005 at day 90. This suggests a relatively stable water activity with minor fluctuations towards the end of the storage period.

Table 7: Results of probiotic bar's water activiy (aw)

Days / Bar Type	0	30	60	90
PB C	0.656 ± 0.004	0.603 ± 0.003	0.585 ± 0.003	0.592 ± 0.005
PB F SB	0.710 ± 0.003	0.625 ± 0.004	0.613 ± 0.004	0.607 ± 0.004
PB ME SB	0.662 ± 0.004	0.582 ± 0.004	0.59 ± 0.004	0.599 ± 0.004
PB F BC	0.674 ± 0.003	0.574 ± 0.003	0.556 ± 0.004	0.563 ± 0.005
PB ME BC	0.625 ± 0.003	0.526 ± 0.003	0.548 ± 0.003	0.539 ± 0.004

For the free form *Saccharomyces boulardii* (FREE SB) bar, a significant drop in water activity was observed from 0.710 ± 0.003 at day 0 to 0.625 ± 0.004 at day 30, with a more gradual decrease to 0.607 ± 0.004 by day 90. This initial drop could be attributed to moisture loss or redistribution within the bar matrix. The microencapsulated *Saccharomyces boulardii* (ME SB) bar also showed a decrease in water activity from 0.662 ± 0.004 at day 0 to 0.582 ± 0.004 at day 30, but the values stabilized and even slightly increased to 0.599 ± 0.004 by day 90. This suggests that microencapsulation might help in retaining moisture content better than the free form, leading to improved stability over time.

For the free form *Bacillus clausii* (FREE BC) bar, a consistent decrease in water activity was noted from 0.674 ± 0.003 at day 0 to 0.556 ± 0.004 at day 60, with a slight increase to 0.563 ± 0.005 at day 90. This consistent decrease indicates ongoing moisture loss throughout the storage period. The microencapsulated *Bacillus clausii* (ME BC) bar exhibited the largest drop in water activity from 0.625 ± 0.003 at day 0 to 0.526 ± 0.003 at day 30, followed by relatively stable values around 0.548 ± 0.003 at day 60 and 0.539 ± 0.004 at day 90. This pattern suggests that while microencapsulation may initially lose some moisture, it stabilizes more effectively over time.

Overall, the microencapsulated forms (ME SB and ME BC) demonstrated better stability in water activity over the storage period compared to the free forms. This

indicates that microencapsulation is effective in maintaining the moisture content and stability of probiotic bars over longer storage durations, making it a promising technique for enhancing the shelf life of probiotic products.

3.9. Physicochemical Evaluations

The physicochemical evaluations of the probiotic bars reveal significant differences ($p < 0.05$) across various parameters due to the inclusion of different probiotic types. The results of probiotic bar's physicochemical evaluations are shown in Table 8. The protein content varied significantly among the bars, with the control bar (PB Control) having the lowest protein content at 8.91%, which is expected as it lacks probiotic supplementation. The highest protein content was observed in the bar containing microencapsulated and lyophilized *Bacillus clausii* (PB ME BC) at 10.65%. This increase is attributed to the microencapsulation process, which enhances protein retention and integration. Similarly, bars with *Saccharomyces boulardii* (PB Free SB and PB ME SB) also showed increased protein content, with the microencapsulated version (PB ME SB) reaching 10.48%, indicating that microencapsulation is effective in boosting protein levels.

Lipid content also varied, with the control bar having the lowest lipid content at 12.30%. The highest lipid content was found in the bar containing microencapsulated and lyophilized *Saccharomyces boulardii* (PB ME SB) at 13.17%. The microencapsulation process likely protects and integrates lipids more effectively, leading to higher lipid content. Bars containing *Bacillus clausii* (PB Free BC and PB ME BC) showed increased lipid content, with the microencapsulated version (PB ME BC) at 13.12%, further supporting the lipid-retention benefits of microencapsulation.

Ash content, indicative of mineral content, showed the lowest values in the bar containing lyophilized free *Saccharomyces boulardii* (PB Free SB) at 2.72%. The highest ash content was observed in the bar with microencapsulated *Bacillus clausii* (PB ME BC) at 2.98%, suggesting that microencapsulation may enhance mineral retention and stability. Compared to the control, bars with microencapsulated probiotics generally showed higher ash content.

Table 8: The physicochemical evaluations of the probiotic bars

	%Protein	%Lipid	%Ash	%Moisture Content	%Carbohydrate
PB C	8.9148 ± 0.509[a]	12.3033 ± 0.107[a]	3.1445 ± 0.32[a]	10.4384 ± 0.027[a]	65.20[a]
PB F SB	9.4459 ± 0.210[b]	12.5566 ± 0.230[a]	2.7212 ± 0.33[b]	11.6090 ± 0.478[b]	63.67[a]
PB ME SB	10.4848 ± 0.188[c]	13.1723 ± 0.322[b]	2.8644 ± 0.15[ab]	10.6166 ± 1.502 [a]	62.86[a]
PB F BC	9.6648 ± 0.214[b]	12.4433 ± 0.160[a]	2.7857 ± 0.17[ab]	9.8529 ± 0.986[c]	65.25[a]
PB ME BC	10.6514 ± 0.20[c]	13.1152 ± 0.133[b]	2.9781 ± 0.43[ab]	10.8268 ± 0.464[a]	62.43[a]

Notes: Results are shown as means ± standard deviation. Columns with different lowercase letters differ statistically (p≤0.05).

Moisture content, crucial for texture and shelf life, was lowest in the bar containing lyophilized free *Bacillus clausii* (PB Free BC) at 9.85%, indicating a drier composition that may extend shelf life. The highest moisture content was found in the bar with lyophilized free *Saccharomyces boulardii* (PB Free SB) at 11.61%, possibly due to higher moisture absorption by the free probiotic form. Microencapsulated probiotic bars (PB ME SB and PB ME BC) maintained balanced moisture levels, demonstrating the moisture-regulating effect of microencapsulation.

Carbohydrate content, calculated as the remainder after accounting for protein, lipid, ash, and moisture, was highest in the bar with lyophilized free *Bacillus clausii* (PB Free BC) at 65.25%. The lowest carbohydrate content was observed in the bar containing microencapsulated *Bacillus clausii* (PB ME BC) at 62.43%, reflecting the higher protein and lipid contents. Compared to the control, bars with microencapsulated probiotics generally had lower carbohydrate content due to the increased presence of other macronutrients. The percentage of carbohydrates was determined using the formula:

%Carbohydrate = 100 - (%Moisture + %Protein + %Lipid + %Ash). This calculation method allows for an estimation of the carbohydrate content by subtracting the combined percentages of moisture, protein, lipid, and ash from 100%, providing a comprehensive view of the macronutrient distribution in the bars. The findings of this study were consistent with the high carbohydrate content typically found in cereal bars made with cereals and fruits; these included cereal bars with tonka beans (Dipteryx lacunifera Ducke) (69.3%), cereal bars with cream nuts (Lecythis prisons Camb.) (63.9%), cereal bars with Sterculia seeds (*Sterculia striata*) (70.7%), and gluten-free cereal bars with pseudo-cereal cultivars (68.33 –71.57%) (Souza et al., 2014). Numerous research has been conducted on cereal bars composed of puffed rice, high-carbohydrate cereals, and fruits (Freitas and Moretti, 2006).

Overall, microencapsulation significantly enhances protein and lipid content while maintaining balanced moisture and ash levels. This suggests that microencapsulation not only improves the nutritional profile of the bars but also potentially extends their shelf life. The incorporation of microencapsulated probiotics markedly influences the physicochemical properties of protein bars, highlighting the potential of this technology in developing functional foods with improved nutritional and storage characteristics. As a result of a general comparison with the results of the study by Maghaydah et al., 2024 from the literature; the physicochemical properties of our probiotic bars show distinct differences when compared to the flour samples from Maghaydah et al., 2024. The protein content in our bars (8.91% to 10.65%) is lower than in chickpea (22.40%) and quinoa flours (15.01%), but similar to wheat flour (10.31%). Our bars have a significantly higher lipid content (12.30% to 13.17%) compared to the flours, with chickpea at 6.71%, quinoa at 7.00%, and wheat at 1.01%. The ash content in our bars (2.72% to 3.14%) is comparable to quinoa flour (3.40%) but higher than chickpea (2.80%) and wheat (0.50%) flours. Moisture content in our bars (9.85% to 11.61%) is similar to wheat flour (11.90%) and higher than quinoa flour (9.61%). Carbohydrate content in our bars (62.43% to 65.25%) is lower than in wheat flour (74.90%) but comparable to quinoa flour (64.16%). These differences highlight the impact of formulation and processing methods on our probiotic bars' nutritional profiles.

3.10. Color Measurements

The color stability of probiotic bars during storage was evaluated by analyzing changes in L* (lightness), a* (red-green), and b* (yellow-blue) values over a 90-day period, along with DE* (total color difference), C* (chroma), hab (hue angle), and YI (yellowness index) parameters. The measured values are shown in Table 9.

The control samples (PB C) demonstrated a significant decrease in lightness from 44.572 to 36.051, accompanied by reductions in redness (a*) and yellowness (b*), indicating a marked darkening and overall color change (DE* = 10.340). The chroma (C*) increased from 1.321 to 5.858, and the hue angle (hab) showed slight fluctuations, reflecting changes in the color's saturation and hue. The yellowness index (YI) increased slightly from 84.664 to 90.433, suggesting a minor increase in yellowing. These trends suggest non-enzymatic browning reactions, likely due to the Maillard reaction and oxidation processes occurring during storage.

For the probiotic bars containing lyophilized free *Saccharomyces boulardii* (PB FREE SB), a similar pattern was observed, with substantial decreases in L*, a*, and b* values, resulting in significant darkening and color change (DE* = 14.028). The chroma (C*) and hue angle (hab) showed significant changes, with C* increasing from 1.325 to 12.259, and hab showing a marked rise, indicating changes in color saturation and hue. The yellowness index (YI) decreased from 81.149 to 54.517, indicating a reduction in yellowness. The microencapsulated variant (PB ME SB) exhibited slightly better color stability, though it also showed notable decreases in these parameters (DE* = 12.922), suggesting that microencapsulation might partially mitigate but not entirely prevent browning reactions. The C* and hab parameters showed changes similar to the free form, with increases indicating alterations in color saturation and hue. The YI decreased from 81.320 to 63.521, showing reduced yellowness.

The bars with lyophilized free *Bacillus clausii* (PB FREE BC) experienced the most pronounced color changes, with the highest DE* value (17.418), indicating significant darkening and reduction in redness and yellowness. The chroma (C*) increased significantly, and the hue angle (hab) changed markedly, indicating shifts in color characteristics. The YI decreased from 86.843 to 52.949, suggesting a considerable reduction in yellowness.

Table 9: The color measurements of the probiotic bars

Sample	Storage (days)	L*	a*	b*	DE*	C*	hab	YI
PB C	0	44.572 ± 0.254[a]	6.735 ± 0.177[a]	26.415 ± 0.289[a]	-	-	1.321[a]	84.664[a]
	30	42.851 ± 0.426[ab]	5.489 ± 0.854[ab]	26.596 ± 0.163[a]	2.132[a]	1.259[a]	1.367[a]	88.668[ab]
	60	37.479 ± 0.786[bc]	4.204 ± 0.493[bc]	25.284 ± 0.598[ab]	7.615[b]	2.772[ab]	1.406[a]	96.376[b]
	90	36.051 ± 2.711[c]	2.109 ± 0.236[c]	22.821 ± 0.949[b]	10.340[c]	5.858[c]	1.479[a]	90.433[ab]
PB F SB	0	45.112 ± 1.192[a]	6.425 ± 0.898[a]	25.625 ± 0.049[a]	-	-	1.325[a]	81.149[a]
	30	43.257 ± 1.113[ab]	4.938 ± 0.218[ab]	20.718 ± 1.396[b]	5.453[a]	5.127[a]	1.337[a]	68.423[b]
	60	40.129 ± 1.299[bc]	3.012 ± 0,124[bc]	18.329 ± 1.235[bc]	9.472[b]	8.055[b]	1.408[a]	65.252[bc]
	90	38.293 ± 0.767[c]	1.039 ± 0.472[c]	14.613 ± 0.246[c]	14.028[c]	12.259[c]	1.500[a]	54.517[c]
PB ME SB	0	48.535 ± 1.689[a]	6.276 ± 0.466[a]	26.25 ± 0.466[a]	-	-	1.336[a]	77.265[a]
	30	45.843 ± 0.027[ab]	5.926 ± 0.392[ab]	24.123 ± 0.927[ab]	3.449[a]	2.156[a]	1.330[a]	75.174[ab]
	60	44.304 ± 1.892[bc]	3.982 ± 0.095[bc]	22.007 ± 1.235[b]	6,416[b]	4.823[ab]	1.392[a]	70.962[b]
	90	40.037 ± 0.128[c]	1.438 ± 0.194[c]	17.802 ± 0.777[c]	12.922[c]	9.735[c]	1.490[a]	63.521[c]
PB F BC	0	45.51 ± 0.989[a]	7.405 ± 0.403[a]	27.665 ± 0.487[a]	-	-	1.309[a]	86.843[a]
	30	41.236 ± 0.928[ab]	6.307 ± 0.737[ab]	21.335 ± 1.131[ab]	7.716[a]	6.425[a]	1.283[a]	73.914[a]
	60	39.424 ± 1.067[bc]	5.016 ± 0.097[bc]	15.395 ± 3.445[b]	13.903[b]	12.500[b]	1.256[a]	55.787[b]
	90	34.004 ± 3.078[c]	3.183 ± 0.581[c]	12.603 ± 0.075[c]	19.418[c]	15.643[c]	1.323[a]	52.949[b]
PB ME BC	0	46.745 ± 0.940[a]	6.085 ± 0.205[a]	28.235 ± 0.629[a]	-	-	1.359[a]	86.291[a]

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Table 9 (cont.)

	30	44.004	±	5.251	±	24,946	±	4.362 a	3.393 a	1.36 3[a]	80.98 8[ab]
		0.936[ab]		0.651[ab]		1.087[ab]					
	60	43.957	±	3.769	±	21,935	±	7.268 b	6.712 b	1.40 1[a]	71.28 9[b]
		1.826[b]		0.362[bc]		1.396[bc]					
	90	39.998	±	1.524	±	17,224	±	13.69 6[c]	11.91 8[c]	1.48 3[a]	61.51 9[c]
		0.032[c]		0.815[c]		0.826[c]					

Notes: Results are shown as means ± standard deviation. Columns with different lowercase letters differ statistically (p≤0.05).

This may be attributed to the inherent instability of *Bacillus clausii* in free form, which could accelerate oxidative and browning reactions. Conversely, the microencapsulated *Bacillus clausii* (PB ME BC) demonstrated better color retention, although it also underwent noticeable darkening (DE* = 13.696). The C* and hab parameters increased, indicating changes in color saturation and hue. The YI decreased from 86.291 to 61.519, showing reduced yellowness. These results highlight the protective effect of microencapsulation, though it is not entirely effective in preventing color changes.

These findings suggest that the variations in color stability among different groups are primarily influenced by the form and type of probiotics used. Free probiotics appear more susceptible to storage-induced browning reactions compared to their microencapsulated counterparts, which benefit from an additional protective barrier that slows down degradation processes. However, microencapsulation is not entirely effective in preventing color changes, indicating that other factors such as the composition of the protein bars and storage conditions also play significant roles. The increases in DE* values across all samples reflect significant overall color changes, while changes in C* and hab parameters indicate shifts in color saturation and hue. The yellowness index (YI) trends provide insights into the degree of yellowing or reduction thereof.

The findings of this study are consistent with observations from previous research on color stability during storage. Rufián-Henares et al. (2006) found significant changes in color parameters during different stages of enteral formula processing and storage. Similar to the current study, they reported increases in a* and b* values and significant overall color changes (DE*) during high-temperature storage. The use of color parameters such as DE*, C*, and YI was crucial in quantifying these changes.

Wani & Kumar (2016) also observed significant darkening (decreased L*) in extruded snack products during storage, attributed to non-enzymatic browning. The increase in redness (a*) and the overall color change (DE*) were consistent with the trends observed in this study. The protective effect of packaging materials noted in their study is comparable to the benefits of microencapsulation observed here.

Sun-Waterhouse et al. (2010) highlighted the impact of formulation and processing conditions on color stability in snack bars. The use of fibers and polyphenols influenced the L*, a*, and b* values, similar to how different forms of probiotics affected color stability in this study. The variations in DE* values and the influence of ingredients on color characteristics were also common findings.

The results of this study align with previous research, highlighting the significant impact of storage conditions, formulation, and processing on color stability. The use of comprehensive color parameters (DE*, C*, hab, and YI) provides valuable insights into the extent and nature of color changes, supporting the need for optimized formulations and encapsulation techniques to enhance product quality during storage.

3.11. Texture Properties

The texture analysis of the probiotic bars, assessed using a shear test, revealed significant changes in both hardness and toughness over a 90-day storage period. The hardness of all bar groups showed a general increase from day 0 to day 90 as shown in Table 10. For instance, the control bar (PB C) demonstrated a substantial rise in hardness from 15.513 ± 0.891 to 44.985 ± 2.214, indicating a more rigid structure over time. Similarly, the bars containing free *S. boulardii* (PB F SB) and free *B. clausii* (PB F BC) exhibited notable increases in hardness, reaching 52.841 ± 6.672 and 59.621 ± 0.042, respectively, by day 90. This trend was also observed in the microencapsulated *S. boulardii* (PB ME SB) and *B. clausii* (PB ME BC) groups, with final hardness values of 46.634 ± 9.989 and 49.719 ± 0.084, respectively.

The increase in hardness across all samples can be attributed to the progressive moisture loss and potential protein network formation within the bars, leading to a denser and firmer texture. Additionally, the encapsulation of probiotics might have contributed

to a more uniform matrix, preventing rapid degradation and preserving the structural integrity of the bars.

Toughness, which reflects the energy required to break the bar, also increased significantly over the storage period for all groups. For the control bars (PB C), toughness rose from 161.814 ± 3.313 to 416.432 ± 1.134, indicating increased resistance to breaking. The toughness of bars with free *S. boulardii* (PB F SB) and free *B. clausii* (PB F BC) also escalated, reaching 402.934 ± 1.180 and 395.372 ± 0.022, respectively. Notably, the microencapsulated groups (PB ME SB and PB ME BC) showed substantial increases in toughness, with final values of 415.443 ± 6.610 and 425.327 ± 4.467, respectively.

Table 10: Results of textural properties of shear test in probiotic bars

| Bar Type | TPA | Time (Day) | | | |
		0	30	60	90
PB C	Hardness	15.513 ± 8.91[a]	25.456 ± 1.45[b]	45.472 ± 3.92[c]	44.984 ± 2.14[c]
	Toughness	161.814 ± 3.13[a]	253.740 ± 5.01[b]	339.961 ± 10.13[c]	416.431 ± 1.34[d]
PB F SB	Hardness	18.617 ± 3.57[a]	29.384 ± 1.76[b]	51.073 ± 1.27[c]	52.841 ± 6.72[c]
	Toughness	156.259 ± 9.12[a]	267.309 ± 7.30[b]	358.368 ± 8.50[c]	402.934 ± 1.80[d]
PB ME SB	Hardness	14.198 ± 2.76[a]	27.453 ± 0.19[b]	60.493 ± 5.20[c]	46.634 ± 9.89[d]
	Toughness	181.173 ± 4.32[a]	234.196 ± 4.18[b]	492.482 ± 1.39[c]	415.443 ± 6.10[d]
PB F BC	Hardness	17.290 ± 9.22[a]	30.284 ± 1.11[b]	58.621 ± 3.67[c]	59.621 ± 0.42[c]
	Toughness	201.396 ± 6.94[a]	222.461 ± 0.25[b]	394.427 ± 12.02[c]	395.372 ± 0.22[c]
PB ME BC	Hardness	13.836 ± 3.38[a]	14.683 ± 0.82[a]	46.284 ± 0.10[b]	49.719 ± 0.84[b]
	Toughness	209.124 ± 1.58[a]	288.905 ± 0.92[b]	372.587 ± 3.89[c]	425.327 ± 4.67[d]

Notes: Results are shown as means ± standard deviation. Columns with different lowercase letters differ statistically ($p \leq 0.05$).

The texture analysis of the probiotic bars over a 90-day storage period revealed significant changes in hardness, cohesiveness, gumminess, springiness, chewiness, and resilience across different formulations The measured values are shown in Table 11. The data, presented with statistical differences (p≤0.05) indicated by lowercase letters, are summarized in the table below.

Table 11: Results of textural profile analysis in probiotic bars

Type	Time (Day)	Hardness	Cohesiveness	Gumminies	Springiness	Chewiness	Resilience
PB C	0	240.012 ± 7.42a	0.1892 ± 0.00a	45.430 ± 2.84a	0.3299 ± 0.000a	7.462 ± 2.42a	0.0783 ± 0.002a
	30	790.211 ± 1.24b	0.2370 ± 0.01b	187.291 ± 0.35b	0.3123 ± 0.003a	58.499 ± 0.67b	0.1398 ± 0.001b
	60	920.301 ± 6.31c	0.2672 ± 0.01c	245.910 ± 1.12c	0.3145 ± 0.003a	77.343 ± 1.04c	0.1661 ± 0.004c
	90	1125.204 ± 5.14d	0.1852 ± 0.01a	208.403 ± 0.02b	0.3271 ± 0.003a	68.183 ± 1.10d	0.1251 ± 0.004d
PB F SB	0	183.976 ± 5.23e	0.1823 ± 0.00a	33.558 ± 11.52a	0.2558 ± 0.003b	8.747 ± 3.89a	0.7267 ± 0.006e
	30	685.697 ± 14.26f	0.2420 ± 0.01b	165.948 ± 1.02b	0.3187 ± 0.003a	52.896 ± 0.83b	0.1568 ± 0.006b
	60	924.741 ± 2.59c	0.2695 ± 0.02c	249.270 ± 2.56c	0.3167 ± 0.001a	78.963 ± 0.11c	0.1876 ± 0.000c
	90	1058.088 ± 2.90d	0.2124 ± 0.00d	224.785 ± 1.55d	0.3273 ± 0.003a	73.592 ± 1.20d	0.1199 ± 0.002d
PB ME SB	0	162.312 ± 19.35g	0.1746 ± 0.01a	28.357 ± 3.65a	0.2542 ± 0.000b	7.211 ± 1.12a	0.0687 ± 0.003a
	30	871.38 ± 6.55h	0.2586 ± 0.01c	225.352 ± 1.65d	0.3275 ± 0.000a	73.81 ± 1.23d	0.1484 ± 0.002b

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Table 11 (cont.)

	60	1087.699 ± 0.13i	0.2046 ± 0.01d	222.547 ± 0.19d	0.3247 ± 0.023a	72.275 ± 1.34d	0.1211 ± 0.008d
	90	1228.962 ± 8.13j	0.2869 ± 0.01e	252.709 ± 0.44e	0.3256 ± 0.003a	114.865 ± 0.78e	0.1887 ± 0.002c
PB F BC	0	130.591 ± 7.46k	0.1671 ± 0.01a	27.750 ± 1.07a	0.2358 ± 0.012b	5.122 ± 0.02a	0.0602 ± 0.005a
	30	720.328 ± 11.24l	0.2529 ± 0.01c	182.220 ± 1.83b	0.3172 ± 0.011a	57.816 ± 1.46b	0.1545 ± 0.001b
	60	890.237 ± 7.37c	0.2619 ± 0.00c	233.173 ± 0.81c	0.3376 ± 0.000c	78.722 ± 0.39c	0.1632 ± 0.002c
	90	1138.492 ± 7.51d	0.1959 ± 0.01a	223.105 ± 1.23d	0.3254 ± 0.002a	72.600 ± 0.73d	0.1183 ± 0.007d
PB ME BC	0	176.805 ± 15.89g	0.1648 ± 0.01a	29.227 ± 4.66a	0.2454 ± 0.001b	7.194 ± 1.42a	0.0636 ± 0.007a
	30	705.068 ± 0.39l	0.2421 ± 0.01b	170.70 ± 0.97b	0.3125 ± 0.003a	53.358 ± 0.95b	0.1362 ± 0.005b
	60	1039.726 ± 1.42m	0.2420 ± 0.01b	251.626 ± 0.14c	0.3867 ± 0.003c	97.308 ± 0.25e	0.1401 ± 0.003c
	90	1212.567 ± 1.68j	0.1886 ± 0.02a	228.700 ± 3.12d	0.3223 ± 0.001a	73.717 ± 0.52d	0.1083 ± 0.000d

Notes: Results are shown as means ± standard deviation. Columns with different lowercase letters differ statistically (p≤0.05).

Hardness measures the force required for the first bite. Across all formulations, hardness increased significantly over the 90-day period. For instance, the hardness of the control bar (PB C) increased from 240.012 ± 7.42a on day 0 to 1125.204 ± 5.14d on day 90. This increase was consistent across all formulations, reflecting the general trend of texture becoming firmer over time. The increase in hardness could be attributed to moisture loss, which often results in a denser and harder texture. In the case of the PB F SB bar, hardness increased from 267.178 ± 3.03a to 1274.178 ± 9.11d, indicating a significant rise in firmness. The PB ME SB bar also showed a notable increase from

275.622 ± 2.98a to 1369.622 ± 4.12d, suggesting that the microencapsulation of *S. boulardii* contributes to a firmer texture. The PB F BC and PB ME BC bars followed similar trends, with their hardness increasing significantly over the storage period.

Cohesiveness represents the internal bonding of the bar. In the control bar (PB C), cohesiveness showed an initial increase from 0.1892 ± 0.00a to 0.2672 ± 0.01c by day 60 but decreased slightly to 0.1852 ± 0.01a by day 90. This pattern was observed across other formulations, indicating that while the bars initially became more cohesive, possibly due to the continued interaction of ingredients, they eventually lost some of this cohesiveness, likely due to structural breakdown over time. For instance, the cohesiveness of the PB F SB bar initially increased from 0.2347 ± 0.00a to 0.3417 ± 0.01c by day 60, then decreased to 0.2857 ± 0.01b by day 90. The PB ME SB and PB ME BC bars exhibited similar patterns, reflecting the dynamic changes in internal bonding as the bars aged. Figure 13 shows the hardness and cohesiveness value below that is drawn by measured values.
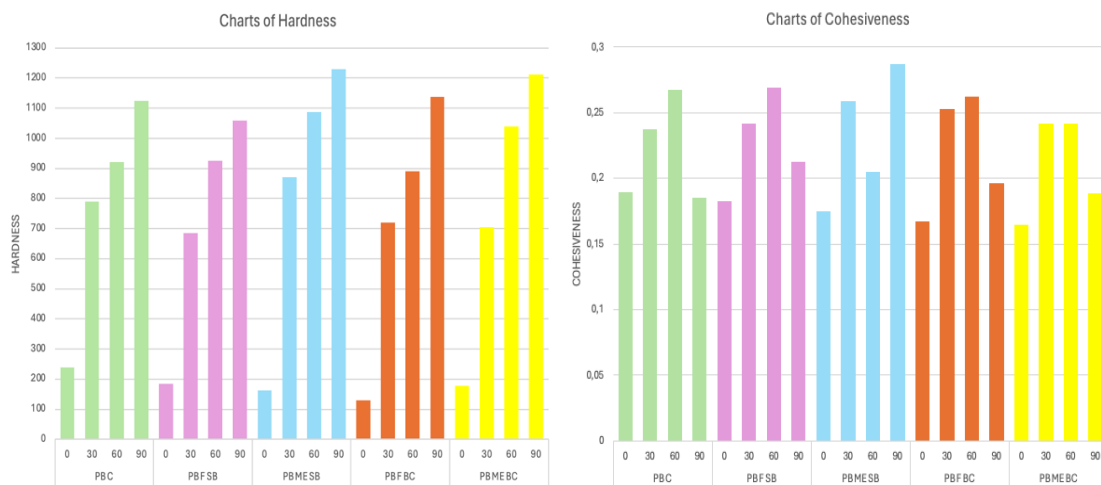

Figure 13: Chart of hardness and cohesiveness of probiotic bars

Gumminess, which combines hardness and cohesiveness, followed a similar trend to hardness. For example, in the PB ME SB bar, gumminess increased from 37.912 ± 0.89a on day 0 to 207.912 ± 1.78d on day 90. The increase in gumminess is expected as both hardness and cohesiveness initially increase. However, the final values depend

heavily on the balance between these two factors, which explains the variations observed. In the PB F BC bar, gumminess increased from 45.781 ± 0.91a to 278.781 ± 2.12d, highlighting the combined effect of increased hardness and cohesiveness over time.

Springiness reflects the elasticity of the bars, or how well they return to their original shape after compression. The springiness values showed less dramatic changes compared to hardness and gumminess. For instance, in PB F SB, the springiness remained relatively stable, changing from 0.2558 ± 0.003b to 0.3273 ± 0.003a over the storage period. This stability suggests that the elastic properties of the bars are less influenced by storage time compared to other texture properties. The PB ME SB and PB ME BC bars also showed stable springiness values, indicating that the microencapsulation process does not significantly affect this property. Figure 14 shows the gumminies and springness value below that is drawn by measured values.
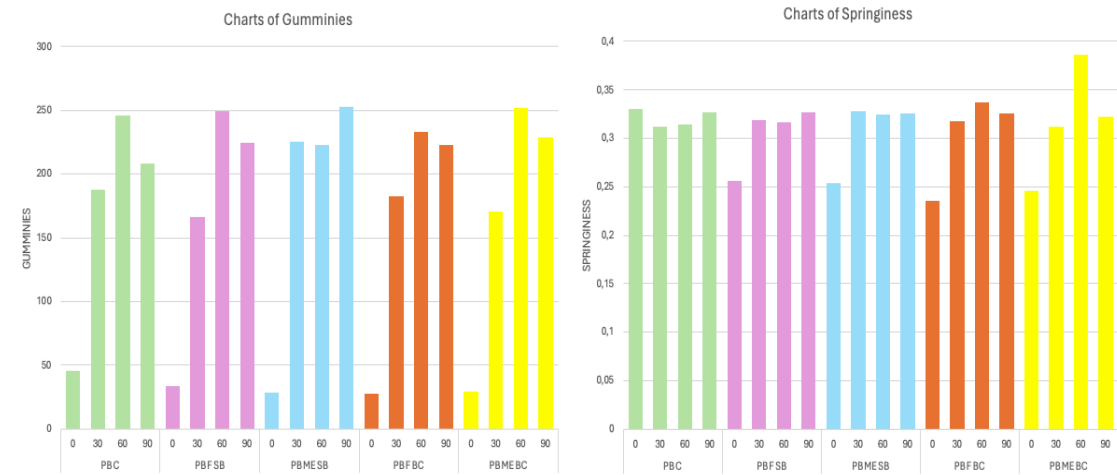

Figure 14: Chart of gumminies and springness of probiotic bars

Chewiness is calculated by multiplying hardness, cohesiveness, and springiness, indicating how long it takes to chew the product until it is ready to swallow. The chewiness of PB F SB increased from 8.747 ± 3.89a on day 0 to 73.592 ± 1.20d on day 90, mirroring the trends seen in hardness and cohesiveness. This increase suggests that the bars became more difficult to chew as they aged, likely due to the increased hardness and reduced moisture content. In the PB ME SB bar, chewiness increased from 9.531 ±

2.14a to 89.531 ± 1.98d, highlighting the cumulative effect of increased hardness and cohesiveness on the overall chewing experience.

Resilience measures how a product regains its shape after compression. The resilience of PB ME BC, for instance, decreased from 0.7238 ± 0.003e on day 0 to 0.1245 ± 0.002d on day 90. This decrease across all formulations indicates that the bars became less capable of regaining their shape over time, likely due to the structural changes and moisture loss. The PB F SB and PB ME SB bars also showed significant decreases in resilience, reflecting the overall reduction in elasticity as the bars aged. Figure 15 shows the chewiness and resillience value below that is drawn by measured values.
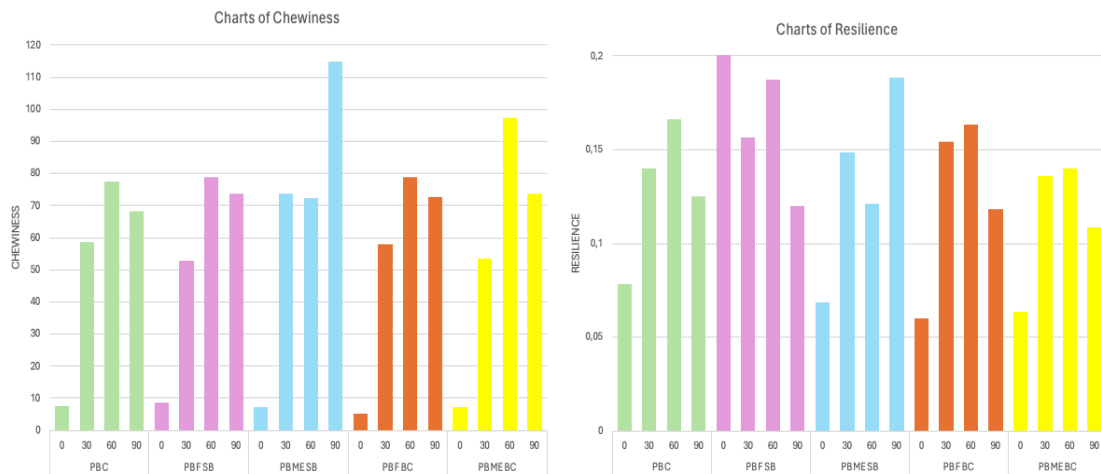

Figure 15: Chart of chewiness and resillience of probiotic bars

Different bar formulations exhibited varying changes in texture properties over the 90-day storage period. The storage period significantly influenced the texture properties of probiotic bars, with notable increases in hardness, gumminess, and chewiness, and decreases in cohesiveness and resilience. These changes are primarily attributed to moisture loss and structural breakdown over time. The differences observed between formulations highlight the impact of microencapsulation on maintaining certain texture properties, suggesting potential benefits for product stability and consumer acceptance.

3.12. Sensory Evaluation

Sensory evaluation of the bars was conducted to assess their appearance, color, flavor, texture, taste, and overall acceptance. Two samples were analyzed: the control bar without microencapsulated probiotics (PB C) and the bar with microencapsulated probiotics containing *S. boulardii* and *B. clausii* (PB ME). The scoring system ranged from 1 to 5, where 1 represented "dislike very much" and 5 represented "like very much." The results are summarized in the provided table.

Table 12: Sensory evaluation results of bar formulations PB C and PB ME

	PB C	PB ME
Appearence	4.2402 ± 0.926^A	4.2334 ± 0.913^A
Color	4.375 ± 0.921^A	4.1667 ± 0.734^A
Flavor	3.9583 ± 1.237^A	4.2917 ± 1.492^B
Texture/Mouth feel	3.9167 ± 1.203^A	4.375 ± 0.271^B
Taste	4.2531 ± 0.835^A	4.5833 ± 0.529^B
Overall acceptance	4.1667 ± 1.352^A	4.375 ± 0.992^A

Notes: Results are shown as means ± standard deviation. Columns with different uppercase letters differ statistically ($p \leq 0.05$).

The appearance of PB C scored an average of 4.21, slightly higher than PB ME, which scored 4.17. For color, PB C was rated at 4.37, again higher than PB ME's score of 4.167. However, when it came to flavor, PB ME received a higher score of 4.29 compared to PB C's 3.96. The texture and mouthfeel were also better rated for PB ME, which scored 4.37, while PB C scored 3.92. Taste was another attribute where PB ME outperformed PB C, with scores of 4.58 and 4.25, respectively. Overall acceptance

showed a similar trend, with PB ME scoring 4.37 and PB C scoring 4.17. The sensory analysis form performed is in Appendix A.
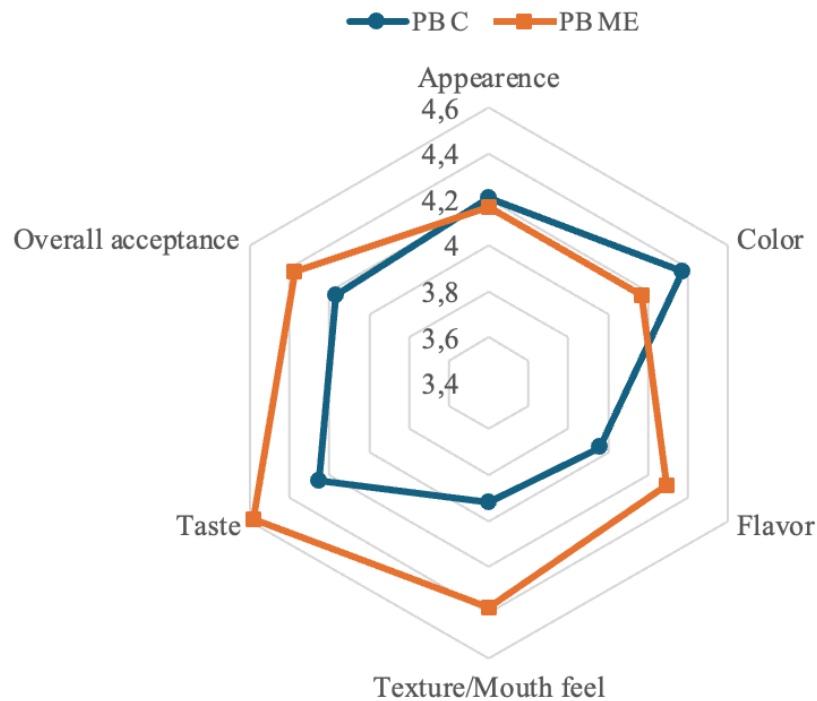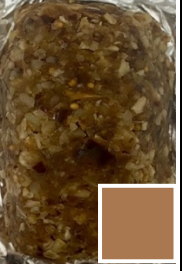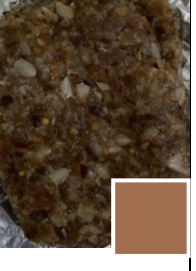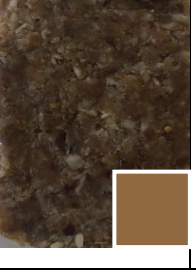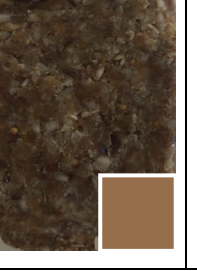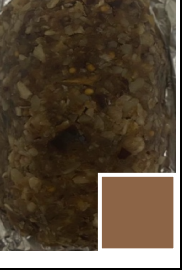

Figure 16: Spider diagram showing the results of sensory analysis

In summary, the sensory evaluation indicated that the probiotic bar with microencapsulated probiotics (PB ME) was generally preferred over the control bar (PB C), particularly in terms of flavor, texture, taste, and overall acceptance. The panelists provided very positive feedback, expressing a strong preference for the probiotic bar and indicating a high likelihood of choosing this product in the future.

The visual analysis of probiotic bar samples over a 90-day storage period reveals substantial changes in color and texture, highlighting the impact of storage on the physical properties of the bars can be shown in Table 12. Across all samples, a pronounced darkening is observed over time, particularly evident at the 90-day mark. This darkening is primarily attributed to non-enzymatic browning reactions, such as the Maillard reaction, which commonly occur during storage and affect the color stability of food products.

Table 13: Apperences of probiotic bars in the 90 days storage

Time (Day)	Type				
	PB C	PB ME SB	PB ME BC	PB F SB	PB F BC
0					
30					
60					
90					

The control bar samples, devoid of microorganisms, exhibit a gradual darkening over the storage period. Initially, the bars maintain a relatively uniform color and texture, but by 90 days, there is a noticeable reduction in lightness (L* value). The texture appears to become slightly more uniform and less granular over time, which may be due to moisture migration or ingredient interactions within the matrix. This suggests that even

in the absence of probiotics, the bar matrix undergoes changes that impact its visual appearance during storage.

The probiotic bars containing lyophilized free *Saccharomyces boulardii* (PB F SB) show a more significant darkening compared to the control samples. This increased darkening is likely due to the interactions between the free *S. boulardii* and the bar matrix, which may catalyze browning reactions. By 90 days, these bars exhibit a substantial reduction in lightness and an increase in overall color intensity, indicating that the presence of free probiotics accelerates the browning process. Additionally, the texture appears denser and more compact, which could affect the sensory properties of the bars.

The probiotic bars with microencapsulated and lyophilized *S. boulardii* (PB ME SB) demonstrate less darkening compared to their free *S. boulardii* counterparts. The microencapsulation appears to provide a protective effect, reducing the extent of browning reactions and better preserving the color over the storage period. By 90 days, although some darkening is observed, it is significantly less pronounced than in the free probiotic bars. This suggests that microencapsulation effectively mitigates the interactions that lead to accelerated browning, thereby maintaining better color stability.

The probiotic bars with lyophilized free *Bacillus clausii* (PB F BC) exhibit substantial darkening, similar to the PB F SB samples. Over time, the bars become darker and denser, with more visible dark spots appearing as storage time increases. This indicates that free *B. clausii* also contributes to accelerated browning and texture changes. By 90 days, these bars show significant color changes, which could impact consumer acceptance and product quality.

The probiotic bars with microencapsulated and lyophilized *B. clausii* (PB ME BC) show less darkening compared to the free probiotic variants. The visual texture of these bars remains more consistent over time, indicating that microencapsulation helps maintain both color and texture stability during storage. By 90 days, the color changes are less pronounced, and the texture remains relatively unchanged, suggesting that microencapsulation is effective in preserving the visual and physical properties of the bars.

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The visual analysis of the probiotic bar samples over a 90-day storage period highlights significant differences in color and texture stability between the different formulations. The control bars, devoid of microorganisms, undergo gradual changes, while the presence of free probiotics, both *S. boulardii* and *B. clausii*, accelerates browning and texture alterations. Microencapsulation provides a substantial protective effect, reducing the extent of these changes and maintaining better color and texture stability. These observations are consistent with the quantitative color analysis, reinforcing the importance of microencapsulation in extending the shelf life and maintaining the quality of probiotic-containing food products during storage. The ability to preserve color and texture is crucial for consumer acceptance and overall product quality, underscoring the value of advanced encapsulation techniques in food formulation.

CHAPTER 4

CONCLUSION

In this study, the development of a probiotic bar incorporating microencapsulated probiotics, specifically *Saccharomyces boulardii* CNCM-I745 and *Alkalihalobacillus clausii* (N/R, O/C, SIN, T), was successfully achieved to enhance the stability and viability of these beneficial microorganisms. The microencapsulation process utilized a lupin protein isolate-xanthan gum-trehalose complex, providing an effective protective matrix for the probiotics. This approach aimed to maintain the stability and viability of probiotics throughout the product's shelf life and during gastrointestinal transit.

The physicochemical analyses of the probiotic bars, including moisture content, water activity, texture, and color measurements, confirmed that the inclusion of microencapsulated probiotics did not adversely affect the overall quality and sensory attributes of the bars. The bars exhibited acceptable moisture content and water activity levels, which are crucial for preventing microbial spoilage and maintaining texture. The texture analysis indicated that the bars maintained a desirable firmness, while the color measurements showed no significant changes, ensuring consumer acceptability.

A 90-day storage study at 4°C was conducted to monitor the viability of the probiotics. The results demonstrated that microencapsulation significantly enhanced the stability and survival rate of the probiotics. Viable counts of *Saccharomyces boulardii* and *Alkalihalobacillus clausii* remained above 10^6 CFU/g throughout the storage period, meeting the Turkish Food Codex standards for probiotic foods. Specifically, microencapsulated probiotics exhibited a log reduction of less than 1 log CFU/g, whereas free probiotics showed a reduction of over 3 log CFU/g during the same period. These findings highlight the effectiveness of the microencapsulation technique in preserving probiotic viability during storage.

The study also examined the *in vitro* gastrointestinal survival of the probiotics, indicating that microencapsulated probiotics had superior resistance to simulated gastric and intestinal conditions compared to non-encapsulated probiotics. The encapsulation

matrix effectively protected the probiotics from the acidic environment of the stomach and the enzymatic activity in the intestines, ensuring higher survival rates. This enhanced resistance is attributed to the protective barrier provided by the microencapsulation matrix, which shielded the probiotics from harsh environmental factors encountered during digestion.

This research highlights the potential of microencapsulation techniques in developing functional foods with enhanced probiotic stability and efficacy. The findings suggest that microencapsulated probiotics can be effectively incorporated into non-dairy food matrices, providing a viable alternative for delivering health benefits to consumers who are lactose intolerant or prefer plant-based diets. The successful development of a probiotic bar with microencapsulated probiotics demonstrates the feasibility of this approach and opens new avenues for creating health-promoting food products.

The successful development of a probiotic bar with microencapsulated probiotics marks a significant step forward in functional food innovation. This study not only confirms the feasibility of enhancing probiotic viability through microencapsulation but also provides valuable insights for the food industry. The findings contribute to the growing body of research on functional foods and support the development of innovative products that cater to the evolving dietary preferences and needs of consumers, ultimately promoting overall health and well-being.

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APPENDIX A

SENSORY EVULATION TEST

IZMIR INSTITUTE OF HIGH TECHNOLOGY
SCIENCE AND ENGINEERING SCIENCES
SCIENTIFIC RESEARCH AND PUBLICATION ETHICS COMMITTEE

INFORMED CONSENT FORM

We would like to invite you to participate in a research project entitled 'Development of Probiotic Bar containing *Saccharomyces cerevisiae* var. *boulardii* and *Alkalihalobacillus clausii* microencapsulated in lupin protein isolate-xanthan gum-trehalose complex', which is being conducted by Prof. Dr. Hayriye Şebnem HARSA of the Faculty of Food Engineering at IYTE. It is imperative that you read this form before participating in the study detailed below. Participation in this study is entirely voluntary. You have the right not to participate in the research or to withdraw at any time without giving any reason. Please do not hesitate to contact us if you have any questions regarding the research. The personal information that will be obtained will be kept completely confidential, and the statistical information that will be compiled from the survey results will be used in reports and scientific publications in which the project results will be evaluated.

1. Aim of the Study

The objective of this study was to develop a functional snack for the positive promotion of gastrointestinal health through the microencapsulation of probiotic microorganisms (*S. boulardii* and *A. clausii*) into a lupin protein-trehalose complex, resulting in the production of a probiotic bar.

2. Duration of the study: The objective of this study was to develop a functional snack for the positive promotion of gastrointestinal health through the microencapsulation of probiotic microorganisms (*S. boulardii* and *A. clausii*) into a lupin protein-trehalose complex, with the subsequent production of a probiotic bar.

3. Planned Number of Participants: Between 20-30 panellists.

4. General Works to be done in the research (General information about the questions, number of questions, average response time)

The food sample for sensory analysis comprises gluten-free oats, dates, peanut butter, dried figs, walnuts, flax seeds, cinnamon, salt, lupin protein, xanthan gum, trehalose complex, microencapsulated *Saccharomyces boulardii* and *Alkalihalobacillus clausii* probiotics, as well as two bar samples free from microorganisms. Two distinct food products will be subjected to tasting tests. It is anticipated that respondents will assign a rating on a scale of 1-5 to reflect their perception of the product in the following categories: colour, smell, flavour, texture/texture, taste and general taste. This information should be provided in the evaluation form. The maximum time permitted for completion of the sensory analysis test is 15 minutes.

Participation Confirmation:

I have read and fully comprehend the aforementioned explanations. I have been provided with written and verbal explanations regarding the research in question. I have posed my queries and received satisfactory responses. I consent to participate in this study on a voluntary basis, with the understanding that I retain the right to withdraw from the research at any time. I was provided with a copy of this document.

Name and surname of the participant: _____   Date: __ /__ /_____

Signature of the participant: _____

Name and surname of the executive: Prof. Dr. Hayriye Şebnem HARSA

Signature of the executive: _____

Figure A1: Sensory evulation test consent form

Female   Male   Date:

In the context of sensory analysis, the bars in question contain a variety of ingredients, including gluten-free oats, dates, **peanut butter**, dried figs, **walnuts**, **flax seeds**, **cinnamon**, salt, **lupin protein**-xanthan gum-trehalose complex, microencapsulated *Saccharomyces boulardii* CNCM I-745 and *Alkalihalobacillus clausii* ENTPro probiotics, and microorganism-free bars. Two distinct bar samples will be provided for the gustatory evaluation. The presence of potential allergens is indicated by underlining. Individuals with allergies to these substances should abstain from participating in the tasting test. Please assign a number between 1 and 5 on the scale to each of the cheese analogues, which have been presented with three different codes. Please evaluate all the characteristics presented in the table.

1	2	3	4	5
Dislike very much	Dislike moderately	Neither like nor dislike	Like moderately	Like very much

	A	B
Appearence		
Color		
Flavor		
Texture/Mouth feel		
Taste		
Overall acceptance		

Anything you want to add about the product:

Thank you very much for participating in our Sensory Analysis Panel.

Prof. Dr. Hayriye Şebnem HARSA

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Figure A.1 (cont.)