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LABORATORY OF FOOD MICROBIOLOGY & BIOTECHNOLOGY

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Postgraduate Diploma Thesis

Selection of probiotic bacteria for food and feed applications

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Επιλογή προβιοτικών βακτηρίων για χρήση σε τρόφιμα και ζωοτροφές

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MSc Food Safety & Quality Management Systems
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Laboratory of Food Microbiology & Biotechnology

Abstract

The purpose of this study is the *in vitro* screening and selection of probiotic bacteria suitable for the formulation of functional foods (food application) or for the development of new feed strategies (feed application) in order to boost human and animal health.

For this reason, strains of *Lactobacillus delbrueckii*, and previously classified as *Lactobacillus* spp., *Lacticaseibacillus rhamnosus, Lactiplantibacillus plantarum* and *Levilactobacillus brevis* isolated from food sources such as olives, sourdoughs, donkey milk, and cheese were screened for their probiotic potential with *in vitro* assays. The tests included anti-microbial activity against pathogenic bacteria (*E. coli, Listeria monocytogenes, Salmonella,* and *S. aureus*) using the agar well diffusion assay. In addition, the selected strains were tested for their ability to produce biofilm using the crystal violet assay, as well as for their potential to inhibit the adhesion of pathogenic yeasts belonging to *Candida* spp., by measuring the biofilm produced in the presence of the strain and comparing it to the biofilm produced in the absence of it. Finally, multivariate analysis was employed to elucidate the performance of the strains and select the best candidates to be used in the formulation of functional foods and also develop new feed strategies. Results revealed that the strains Ldb2, Ldb3, Lbr7, Lbr11, Lpl10, and Lpl11, isolated from *L. plantarum*, *L. delbrueckii*, and *L. brevis*, demonstrated strong antimicrobial effects against a variety of pathogens, including *C. tropicalis*, *C. albicans*, *C. glabrata*, *E. coli* ATCC 35218, *E. coli* ATCC 105393, *Salmonella* spp., and *S. aureus* DSM 1104. These findings suggest their potential as effective candidates for probiotics. Both *L. delbrueckii* and *L. plantarum* formed strong biofilms against *E. coli* ATCC 35218, *E. coli* ATCC 105393, *Salmonella* spp., and *S. aureus* DSM 1104, while *L. plantarum* also exhibited significant inhibition of *C. tropicalis* and *C. albicans*. Additionally, *L. plantarum* and *L. rhamnosus*, specifically the strains Lpl5, Lpl6, Lrh5, and Lrh7, demonstrated effective inhibition of *Listeria monocytogenes* growth. These findings provide valuable insights into the probiotic and

antimicrobial properties of various, with Ldb2, Ldb3, Lbr7, Lbr11, Lpl10, and Lpl11 emerging as promising candidates for further investigation and potential use in probiotic formulations aimed at combating diverse pathogens in food and feed applications.

Scientific area: Food Microbiology

Key words: probiotics, food and feed application, *Candida* species, pathogens, biofilm formation, adhesion, anti-microbial activity

Επιλογή προβιοτικών βακτηρίων για χρήση σε τρόφιμα και ζωοτροφές

ΠΜΣ Συστήματα Διαχείρισης Ποιότητας & Ασφάλειας Τροφίμων
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Περίληψη

Ο σκοπός της μελέτης ήταν ο *in vitro* έλεγχος και η επιλογή βακτηρίων με προβιοτικό δυναμικό, κατάλληλων για τη δημιουργία λειτουργικών τροφίμων ή για την ανάπτυξη νέων ζωοτροφών, με σκοπό την ενίσχυση της υγείας του ανθρώπου και των ζώων.

Για το σκοπό αυτό, στελέχη που ανήκουν στο είδος *Lactobacillus delbrueckii* και στελέχη που ταξινομούνταν παλαιότερα στο γένος *Lactobacillus,* όπως *Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum* and *Levilactobacillus brevis*, τα οποία απομονώθηκαν από επιτραπέζιες ελιές, ζυμάρι, γάλα όνου και τυρί, υποβλήθηκαν σε μια σειρά *in vitro* δοκιμών προκειμένου να ελεγχθεί το προβιοτικό τους δυναμικό. Οι δοκιμές περιλάμβαναν αντιμικροβιακή δραστηριότητα έναντι παθογόνων βακτηρίων (*Escherichia coli, Listeria monocytogenes, Salmonella* spp. και *Staphylococcus aureus*) χρησιμοποιώντας τη μέθοδο της μέτρησης της ζώνης αναστολής σε τρυβλία, καθώς επίσης και την ικανότητα των επιλεγμένων στελεχών να δημιουργούν βιοϋμένιο, χρησιμοποιώντας τη μέθοδο του κρυσταλλικού ιώδους. Επίσης μελετήθηκε η δυνατότητα των εν λόγω στελεχών να αναστείλουν την προσκόλληση παθογόνων μυκήτων του γένους *Candida*, μετρώντας το βιοϋμένιο που παράγεται με/χωρίς την παρουσία των επιλεγμένων οξυγαλακτικών βακτηρίων. Τέλος, χρησιμοποιήθηκε πολυμεταβλητή στατιστική ανάλυση που βασίστηκε στη μέθοδο της ανάλυσης κατά συστάδες (cluster analysis) για την επιλογή των καλύτερων στελεχών με σκοπό τη δημιουργία λειτουργικών τροφίμων και ζωοτροφών. Τα αποτελέσματα έδειξαν ότι τα στελέχη Ldb2, Ldb3, Lbr7, Lbr11, Lpl10 και Lpl11, που απομονώθηκαν από τα βακτήρια *L. plantarum*, *L. delbrueckii* και *L. brevis*, παρουσίασαν ισχυρή αντιμικροβιακή δράση έναντι διαφόρων παθογόνων, συμπεριλαμβανομένων των *C. tropicalis*, *C. albicans*, *C. glabrata*, *E. coli* ATCC 35218, *E. coli* ATCC 105393, *Salmonella* spp. και *S. aureus* DSM 1104. Τα αποτελέσματα αυτά ενισχύουν τη δυνατότητα χρήσης των συγκεκριμένων βακτηρίων ως προβιοτικά. Τα βακτήρια *L. delbrueckii* και *L. plantarum* δημιούργησαν ισχυρά βιοϋμένια εναντίον των παθογόνων

E. coli ATCC 35218, *E. coli* ATCC 105393, *Salmonella* spp. και *S. aureus* DSM 1104, ενώ το βακτήριο *L. plantarum* έδειξε επίσης σημαντική αναστολή των παθογόνων *C. tropicalis* και *C. albicans*. Επιπλέον, τα είδη *L. plantarum* και *L. rhamnosus*, ειδικότερα τα στελέχη Lpl5, Lpl6, Lrh5 και Lrh7, παρουσίασαν αποτελεσματική αναστολή της ανάπτυξης του παθογόνου *Listeria monocytogenes*. Αυτά τα ευρήματα παρέχουν πολύτιμες γνώσεις για τις προβιοτικές και αντιμικροβιακές ιδιότητες διαφόρων βακτηρίων, με τα στελέχη Ldb2, Ldb3, Lbr7, Lbr11, Lpl10 και Lpl11 να εμφανίζονται ως υποσχόμενοι υποψήφιοι για περαιτέρω έρευνα και δυνητική χρήση ως προβιοτικά που στοχεύουν στην καταπολέμηση διαφόρων παθογόνων βακτηρίων σε εφαρμογές τροφίμων και ζωοτροφών.

Επιστημονική περιοχή: Μικροβιολογία τροφίμων

Λέξεις κλειδιά: προβιοτικά, εφαρμογή τροφίμων και ζωοτροφών, *Candida* spp., παθογόνοι μικροοργανισμοί, βιοϋμένιο, προσκόλληση, αντιμικροβιακή δραστηριότητα

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With my permission, this paper was checked by the Examination Committee through the plagiarism detection software available at the Agricultural University of Athens, and its validity and originality were cross-checked.

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1. Introduction

Extensive research has been conducted on probiotics, affirming their positive impacts on human health, particularly within the gastrointestinal tract. Scientific literature consistently validates the beneficial effects of probiotics, which can be incorporated into the diet through sources like fermented dairy products (e.g., yogurt, kefir, fermented milk, and cheese), non-dairy items such as bread and bakery products, and certain vegetables like olives. Additionally, probiotics are available in the form of pharmaceutical supplements (Bodke et al., 2022).

According to FAO probiotics are *live microorganisms which provide benefits when consumed in adequate quantities, improving or restoring the gut microflora.* These microorganisms serve as valuable allies in promoting overall health, effectively safeguarding the body. Probiotics have demonstrated their ability to alleviate symptoms of irritable bowel syndrome, prevent vaginal infections, and offer protection against intestinal cancer. Recent studies have suggested potential roles for probiotics in preventing cardiovascular diseases (Dixon et al., 2020) and colorectal cancer (Pino et al., 2020). Commonly referred to as "good bacteria," they naturally inhabit the human gut microbiota in a healthy state, exhibiting resilience to gastric juices and bile salts. Owing to their capacity to adhere to intestinal cells, along with their resistance to digestive challenges, probiotics play a crucial role in modulating intestinal balance and fortifying immune defenses by stimulating the activity of intestinal lymphatic tissues. The evolving understanding of the intestinal microbiota underscores the significance of maintaining a proper balance for improving quality of life and preventing diseases (Lee et al., 2014).

While probiotic strains have traditionally targeted pathogenic gastrointestinal bacteria, there has been a notable rise in infections caused by *Candida* spp. in recent decades. Human exposure to fungal microorganisms is constant, and under normal health conditions, it poses no inherent risks. However, individuals with compromised immune systems are more susceptible to fungal infections. *Candida*, a yeast genus typically exists as a commensal in various areas of the human body. Yet, under specific conditions, it can become pathogenic, forming protrusions from its membrane and transitioning into a hyphal state. In this state, *Candida* spp. can attach to underlying

tissues, becoming opportunistic and causing superficial infections of the skin and mucous membranes, as well as systemic infections.

Managing Candidiasis presents a significant medical challenge due to the increasing number of registered cases. Various *Candida* species have demonstrated the ability to form biofilms, complex aggregations of live and dead bacterial cells adhering to surfaces within a matrix of polysaccharides, extracellular DNA, and proteins. While *Candida* is a common yeast in the human microbiota, specific circumstances such as pH alterations, dysbiosis, and compromised immune responses can transform it into a dangerous pathogen.

The *Candida* genus encompasses over 150 species, with approximately ten posing potential pathogenicity to humans (Kreulen et al., 2023). *Candida albicans*, though the primary pathogen, is joined by *Candida krusei*, *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, and *Candida guillermondi* as noteworthy pathogens. These species, especially *Candida albicans*, naturally inhabit the mucous surfaces of the oral cavity, female genital tract, gastrointestinal tract, and rectal area in a healthy individual. Notably, *Candida* is rarely found on the skin of a healthy subject, except in specific anatomical areas like the armpit and groin, where the juxtaposition of distinct skin areas creates a conducive, moist environment for *Candida* growth (Leite-Jr et al., 2023).

1.1 Probiotics

According to the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO), probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit on the host”. The positive effects on health have primarily been established for particular strains of bacteria, including genera such as *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, S*treptococcus*, *Pediococcus*, *Leuconostoc*, *Bacillus*, and *Escherichia*. In contrast, the only yeast genus with demonstrated efficacy in double-blind studies is Saccharomyces.

Probiotics exhibit the capability to thrive at 37 ◦C, endure adverse conditions within the human digestive tract (such as digestive enzymes, pancreatic juice, and low pH), and promote the well-being of the host environment. They achieve this by regulating

the microbiota, performing various biological functions, and, in some cases, adhering to the mucus of gut epithelial cells (Staniszewski et al., 2021).

Lactic acid bacteria (LAB) play a significant role in the food, dairy, probiotic, and beverage manufacturing sectors. Classified as Generally Regarded as Safe (GRAS), LAB possesses distinct attributes that make them well-suited for diverse applications (Zhang et al., 2020). *Lactobacillus*, a specific type of LAB, is widely employed in the food industry to create primary or starter cultures for various dairy products. Notably, LAB has garnered attention for its ability to modulate the human host system, offering protection against foodborne pathogens. Consequently, there is ongoing exploration of these bacteria's potential as bio-preservatives in the food and dairy industries and as alternatives to antibiotics in medical treatments (Lashani et al., 2020).

Probiotics, especially LAB, have demonstrated efficacy in managing diverse health issues such as inflammatory bowel disease, irritable bowel syndrome, constipation, antibiotic-associated diarrhea, acute diarrhea, allergies, hypertension, and diabetes. However, for probiotic strains to perform at their best, they need specific advantageous characteristics. These include resilience to gastrointestinal conditions, ability to adhere to epithelial cells, capability to absorb cholesterol, hydrolysis of bile salts, protection against virulence genes, non-hemolytic activity, sensitivity to antibiotics, antibacterial qualities, and maintaining viability throughout fermentation and storage (Rashed et al., 2022).

1.2 Bacteria species used as probiotics

Lactic acid bacteria (LAB), notably *Lactobacillus* and *Bifidobacterium*, are the probiotics most extensively researched. The majority of *Lactobacillus* species are regular, non-pathogenic inhabitants in the human and animal intestines, playing a crucial role in sustaining the intestinal microbial ecosystem. LAB have demonstrated the ability to inhibit the proliferation of enteropathogens, showing strong competitiveness primarily through the production of various antimicrobial compounds (Verdenelli et al., 2009).

Lacticaseibacillus rhamnosus, previously classified as *Lactobacillus rhamnosus* stands out as one of the extensively studied and widely utilized probiotic

microorganisms, supported by substantial clinical evidence and recognized for its health advantages, particularly in the gastrointestinal well-being (Lebeer et al., 2018). *L. rhamnosus* has exhibited *in vitro* inhibition against common potentially harmful microorganisms, with a notable impact on suppressing the growth of *C. albicans*. Consequently, this bacterial strain holds promise for enhancing the body's defense against *Candida* infections (Verdenelli et al., 2009). Studies have also demonstrated that this particular species can hinder urogenital and gastrointestinal pathogens through the production of L-lactic acid, hydrogen peroxide, and bacteriocins. This leads to an enhancement of the barrier function of epithelial cells (Reid et al., 2006). Moreover, as indicated by Köhler and colleagues (2012), certain strains of *L. rhamnosus* possess the ability to restrain the formation of biofilm by *C. albicans* on non-living surfaces by suppressing the genes associated with the yeast's biofilm formation.

Lactobacillus crispatus has been identified as a promoter of stability in the typical vaginal microbiota. In the study of Abramov et al (2014) involving healthy women with a recurring history of urinary tract infections, *Lactobacillus* crispatus was administered as a vaginal suppository. This study highlights the capacity of vaginal *L. crispatus* to colonize, modulate the innate immune response in cervicovaginal epithelial cells, and impede the growth of specific pathogens. The biofilm produced by *Lactobacillus* crispatus provides protective advantages to epithelial cells, shielding them from inflammation and influencing the virulence against *Candida* albicans (Wang et al., 2017).

Lactobacillus delbrueckii has a rich history in the fermented milk industry and boasts various biological functions including antibacterial, antioxidant, anticancer properties, and the ability to reduce the adhesion of pathogenic bacteria (Shalabi et al., 2022). As a member of the LAB group, *L. delbrueckii* is widely utilized in commercial settings for its diverse probiotic functions in fermented milk, fermented tofu, and other fermented foods (Yanet al., 2020). The combination of its industrial significance and probiotic potential has attracted considerable attention from researchers. In a study by Tang et al. (2023) involving phenotypic experiments, *Lactobacillus delbrueckii* strain DMLD-H1 exhibited notable antioxidant properties, gastrointestinal tolerance, antimicrobial capabilities, and self-adhesion. Crucially, DMLD-H1, identified as a potential probiotic strain, demonstrated excellent probiotic attributes, indicating its potential to hinder the attachment of pathogenic bacteria and uphold intestinal flora

balance. In more details, the supernatant derived from DMLD-H1 displayed significant bacterial inhibition, particularly against *E. coli* ATCC25922, with higher efficacy than the fermentation broth.

Lactobacillus gasseri is classified as facultative anaerobic LAB and is considered a safe food additive by the US Food and Drug Administration. It is widely present in the human oral cavity, gastrointestinal tract, and female reproductive tract. *L. gasseri* demonstrates diverse probiotic characteristics, including the regulation of intestinal flora, anti-inflammatory and antibacterial properties, preservation of the balance of female vaginal flora, and reduction of uric acid. These attributes suggest its potential as a probiotic candidate (Wu et al., 2023).

Levilactobacillus brevis has demonstrated noteworthy probiotic attributes, notably showing excellent resilience in both acidic and bile conditions. Additionally, *Levilactobacillus brevis* exhibited the capability to utilize a diverse array of carbon sources, including glucose, xylose, arabinose, mannose, galactose, cellobiose, xylitol, arabitol, and lactose, while producing acid. These characteristics make it a desirable choice in the food industry as a probiotic. Moreover, *Levilactobacillus brevis* is able to colonize and attach to epithelial cells and mucosal surfaces, as highlighted by studies such as Lee and Salminen (1995) and Jacobsen et al. (1999). This ability enables them to withstand variations in their intestinal levels and counteract the attachment of pathogenic bacteria through competitive adhesion across the entire intestine, thereby mitigating inflammatory reactions. Hence, *Leviactobacillus brevis* serves to competitively inhibit the presence of pathogenic microbes within the colonic environment (Somashekaraiah et al., 2021).

Lactiplantibacillus plantarum is valued as a functional microorganism, appreciated for its acknowledged health benefits such as survival in the gastrointestinal tract, adhesion capabilities, antioxidant capacity, antimicrobial activity, and modulation of the intestinal microbiota. Moreover, its ability to enhance the nutritional and sensory qualities of specific foods and prolong the shelf-life of fermented products adds to its appeal. Due to these characteristics, *L. plantarum* is extensively utilized as a probiotic culture across a diverse range of food products, with certain strains being promising in the food industry for the development of innovative and functional products (Echegaray et al., 2023).

1.3 Application of probiotic species in food

In recent years, consumers with a focus on health have been actively seeking foods that not only offer nutritional benefits but also possess functional properties that promote health. Consequently, there has been a substantial surge in the demand for functional probiotic foods (Cizeikiene et al., 2021).

Probiotic bacteria commonly used in applications for both animal and human health predominantly fall into two main groups: bifidobacteria and LAB. The term LAB encompasses a phylogenetically homogeneous group within the order *Lactobacillales*, encompassing environmental organisms, members of plant microbiota, commensals found in humans and animals, as well as opportunistic or obligate pathogenic organisms (Chaves et al., 2017).

When aiming to enhance human health, the preference for live probiotic bacteria in food or feed over supplements in pill form may arise. This inclination is rooted in the fact that foods can offer a buffered environment for probiotic microorganisms as they traverse the gastrointestinal tract. Additionally, foods supply essential components that help sustain the viability and effectiveness of probiotics. Furthermore, the synergistic effects of food components can contribute to the multiplication of probiotic bacteria (Cizeikiene et al., 2021).

In the development of novel functional probiotic products, a critical factor is the careful selection of appropriate cultures (Terpou et al., 2019). Each chosen probiotic must meet specific criteria, being safe, capable of surviving the gastrointestinal tract environment, possessing beneficial properties, and being effectively utilized (WHO & FAO, 2016). Probiotic microorganisms intended for use in foods not only need to endure the digestive tract but also must have the ability to multiply in the gut, withstand gastric acidity, and survive in the presence of bile salts. However, the increasing acidity levels can adversely affect the viability of some probiotics (Sahadeva et al., 2011). The survival of probiotic strains in the gastrointestinal tract post-ingestion also hinges on their ability to resist the antimicrobial action of bile salts. Bile tolerance is strain-dependent, and it stands out as one of the most crucial features for probiotic bacteria, indicating that different strains may exhibit distinct behavior and functionality (Masco et al., 2007).

Probiotic bacteria in the gastrointestinal tract not only need to survive but should also possess additional functional properties. Those with high antioxidant and antiproliferative activities, for instance, can contribute to preventing illnesses associated with oxidative stress and reducing the risk of breast and colon cancer (Amaretti et al., 2013). Lactic acid bacteria with antimicrobial activity are valuable not just as biopreservatives in the food industry but also for potential medicinal applications as antimicrobial agents against bacteria-caused infections (Cizeikiene et al., 2021).

The development of probiotics has indeed been historically focused on pharmaceutical applications, targeting conditions such as diarrhea, antibiotic-associated diarrhea, gastrointestinal infections, and chronic inflammation. These applications are rooted in the potential of probiotics to modulate the gut microbiota and contribute to overall health.

While specific probiotics have demonstrated beneficial effects in treating and preventing various health disorders, there are challenges in extending these effects to functional foods for the general population. Functional foods are those that provide health benefits beyond basic nutrition and are typically consumed as part of a regular diet.

One significant challenge lies in demonstrating the long-term effects of probiotic foods, which is a requirement for health claims in certain regions, such as Europe. Conducting large trials over extended durations can be logistically and financially demanding, posing a challenge, especially for smaller laboratories and food companies.

To address this issue, there is a need to identify and validate risk factors for diseases and biomarkers of health. By better understanding these factors, researchers and food companies can design more targeted studies that efficiently assess the long-term effects of probiotic foods. This approach can help streamline the research process and provide valuable insights into the health benefits of probiotics for the broader population (Jankovic et al., 2020).

The oldest recognized advantage of probiotics is the restoration of microbiota balance, commonly understood as an increase in LAB and a decrease in potentially harmful bacteria. Over the past two decades, research has demonstrated the transient modification of the gut microbiota in favor of LAB species in healthy individuals upon the consumption of certain probiotics. Studies have also shown that infants fed probiotic

infant formula exhibit fecal levels of bifidobacteria similar to those of breastfed infants (Langhendries et al., 1995). Despite these findings, linking such changes to specific benefits in the general population remains challenging. However, it is well-established that imbalances in the microbiota are associated with conditions like chronic inflammatory disorders (Manichanh et al., 2006), obesity (Ley et al., 2006), and allergies (Penders et al., 2007). Notably, in Crohn's disease, a reduction in the overall biodiversity of intestinal bacteria has been observed, highlighting the impact of microbiota dysbiosis on health in specific conditions (Ley et al., 2006).

1.4 Application of probiotic species in animal feed

The interest in utilizing *Lactobacillus* spp. has experienced significant growth, not only in the production of functional foods but also in feed production. The primary motivation for incorporating probiotics into feed lies in the pursuit of health-promoting effects similar to the growth stimulators that were previously based on antibiotics, which were prohibited in 2006 (Cizeikiene et al., 2021). Traditionally, probiotics have served as a substitute for low-dose antibiotics in animal applications. The predominant use in animals revolves around enhancing immune function and mitigating the colonization of pathogenic bacteria. The overarching goal is to boost animal production outcomes (Chaves et al., 2017).

In animal nutrition, the utilization of microorganisms as probiotics has demonstrated a confirmed positive impact on the gut microflora. The incorporation of probiotics into animal feed has been shown to notably enhance feed intake, feed conversion ratio, daily weight gain, and overall body weight in a variety of animals such as pig, chicken, sheep, goat, cattle and equines (Samli et al., 2007).

As illustrated in Table 1, the administration of a probiotic not only reduced leg weakness in broilers (Plavinik et al., 1980) but also prevented starvation and sterility in young sows (Bohmer et al., 2006). A study by Mudgal and Baghel (2010) demonstrated that incorporating *Lactobacillus acidophilus* into the diet of buffalo calves led to enhanced calf growth, with a notable increase from 142 g/day in the control group to 207 g/day in the treatment group. Additionally, a pig diet enriched with fructose oligosaccharide and laminarin resulted in improved weight gain and feed conversion

ratio (Miguel et al., 2002). Xu and Gordon (2003) reported that diets containing 0.4% fructo oligosaccharides showed significant enhancements in average daily gain and feed efficiency compared to control diets. Moreover, in commercial settings, combining probiotics and prebiotics with turmeric in broiler diets proved more effective in increasing daily weight gain and feed efficiency than administering prebiotics or probiotics alone (Kumar et al., 2005).

Table 1. Incorporation of probiotic microorganisms into animal feed.

Microorganism	Animal	Effect	Reference
Brewer's yeast	Broilers	Reduction of leg weakness	Plavinik & Scott (1980)
Enterococcus faecium	Young sows	Prevention of starvation and sterility	Bohmer et al (2006)
L. acidophilus	Buffalo calves	Body weight growth	Mudgal & Baghel (2010)
Saccharomyces cerevisiae	Piglets	Improvement of weight gain and feed conversion ratio	Miguel et al (2002)
Lactic acid bacteria	Pigs	Augmentation of carcass output and water capacity	Ceslovas et al. (2005)
Bacilli	Rabbits	Reduction in morbidity and mortality rate	Paulius et al. (2006)
Saccharomyces cerevisiae	Cows	Increasement in milk production	Yu et al. (1997)
Bacilli	Hens	Enhancement of egg quality and production	Kurtoglu et al. (2004)
Saccharomyces cerevisiae	Quails	Improvement in hatchability	Kocaoğlu (2011)

As far as meat production is concerned, there is a growing demand for safe and high-quality meat in the current market. Producers are increasingly inclined towards employing natural and non-chemical supplements that have a positive impact on animal health, enhance productivity, and improve product quality. The utilization of probiotics has been shown to augment carcass output and water-holding capacity, while reducing meat hardness (Ceslovas et al., 2005). Additionally, probiotics have demonstrated a capacity to lower morbidity and mortality rates among growing rabbits during the fattening periods (Paulius et al., 2006). Various probiotic strains, including lactic acid-producing bacteria like *Lactobacillus*, *Pediococcus*, and *Streptococcus*, have been employed in the production of fermented sausages (Vandana et al., 2013).

Adding probiotics to animal feed also has a positive impact on future milk production, as well as fat and protein levels. For instance, supplementing with Saccharomyces cerevisiae has led to increased milk production in dairy cows (Yu et al., 1997), primarily attributed to a rise in cellulolytic bacteria, enhanced fiber degradation, and alterations in rumen volatile fatty acids. In traditional milk products, microbes are

chosen based on their capacity to grow and generate organic acids in milk. When it comes to probiotics, microbes are primarily selected for their potential health-related properties (Vandana et al., 2013).

Supplementing feed with probiotics has been shown to enhance egg quality and production, lower triglycerides and plasma cholesterol, and minimize egg contamination (Kurtoglu et al., 2004). In another study, Kockaglu (2011) observed that both probiotic and prebiotic supplementation led to an improvement in hatchability in quails.

1.5 Biofilm formation

Microorganisms can exist independently or form biofilms, which are organized groups living within a self-produced extracellular polymeric substance (EPS) matrix, adhering to surfaces. Formation of biofilms occurs through the attachment of planktonic microorganisms to surfaces, both biotic and abiotic (Kostakioti et al., 2013). The multicellular nature of biofilms promotes extended survival in diverse environmental conditions. Within biofilms, the "multicellular lifestyle" enhances gene transfer, cooperation, and stratification among microorganisms (Kostakioti et al., 2013). Biofilms are clusters of microorganisms, comprising single or multiple microbial species, densely populated and engaged in intricate social interactions within and between species (Li et al., 2012). Biofilms exhibit distinct growth rates and gene expressions compared to their planktonic counterparts (Lohse et al., 2018). The evolution of microorganisms to create a protective cover, through biofilm formation, serves to establish connections with hosts, resist harsh external conditions, and withstand antibiotics and environmental cues (Castiblanco et al., 2016). Biofilm formation is a common phenomenon in various bacteria, aiding in resilience against challenging environmental factors like pH fluctuations, oxygen radicals, biocides, nutrient scarcity, and antimicrobial agents (Kostakioti et al., 2013). The process of biofilm formation plays a role in the development of antibiotic resistance and the creation of persistent cells, contributing to the challenging persistence of microbial infections (Pang et al., 2018). Biofilms manifest in various pathologies and are widespread, inhabiting medical implants, tissues, water channels, pipes, hospital

surfaces, food processing units, and other surfaces (Donelli et al., 2014). The transition from planktonic growth to biofilm involves intricate regulatory networks that interpret signals, leading to changes in gene expression and subsequent spatial and temporal reorganization of bacterial cells (Kostakioti et al., 2013).

Biofilm-associated microorganisms undergo changes in phenotype and gene expression, displaying resistance to antibiotics, reduced metabolic activity, slowed growth rates, and the production of virulence-associated factors (Gupta et al., 2016). According to the National Institutes of Health (NIH) reports, approximately 65% of microbial infections and 80% of chronic infections result from microbial biofilms, affecting both tissues and medically implanted devices. Moreover, various segments of the food sector, such as poultry, dairy, ready-to-eat products, aquaculture, and others, face significant challenges due to the presence of microorganisms that produce biofilms. This leads to issues like food spoilage, disease outbreaks, and fatalities (Giaouris et al., 2018).

The formation of biofilms is an intricate and multi-step process, encompassing the shift of bacteria from a freely moving planktonic state to a sessile form dedicated to biofilm production. External factors, including temperature, pH, gravitational and hydrodynamic forces, Brownian movements, the characteristics of residing surfaces, quorum sensing, secondary messengers, and various signaling molecules, all play a role in influencing the entire formation process (Zhao et al., 2017). Illustrated in Figure 1, the various phases of biofilm formation can be categorized into four principal steps.

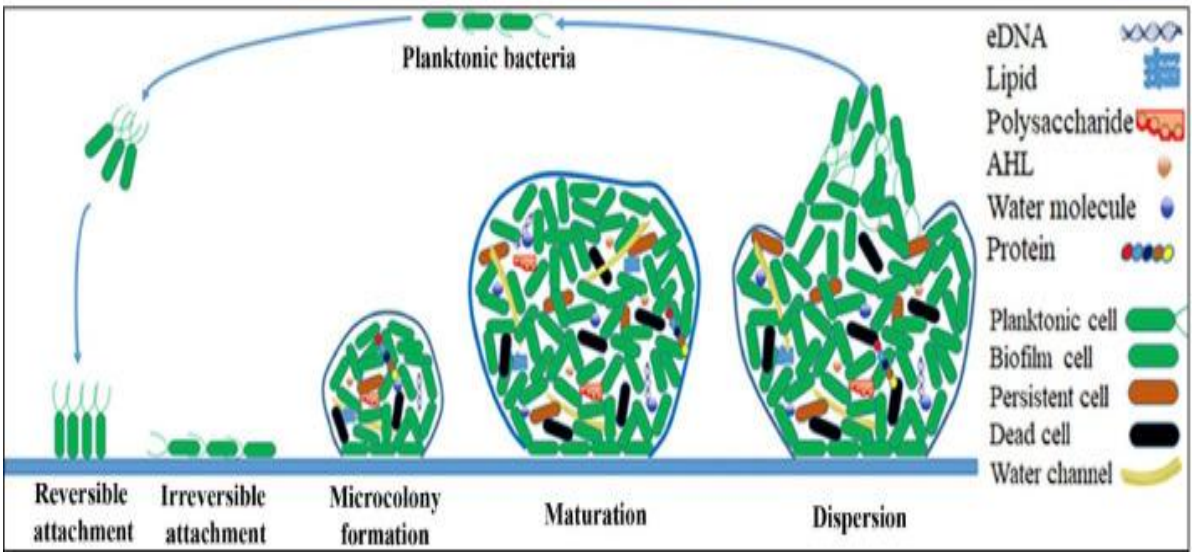

Figure 1. Graphic representation of bacterial biofilm formation (Rather et al., 2021).

- Reversible/Irreversible attachment, the first stage where microorganisms make reversible contact with a surface and irreversible attachment, the subsequent phase characterized by the formation of an irreversible extracellular mono-layer matrix composed of polysaccharides, cell debris, nucleic acids, and proteins.
- Growth or microcolony formation, where cells start to reproduce.
- Maturation, by which entails the development of a fully grown biofilm, exhibiting a three-dimensional architecture.
- Dispersion, detachment and dispersion of cells from the biofilm along with the process of establishing a new biofilm is observed.

The process of initiating biofilm formation begins when planktonic microorganisms adhere to surfaces, marking a crucial stage in transitioning free-moving microorganisms into an organized community structure (Haggag, 2010). In the initial phase, microorganisms attach loosely and reversibly to surfaces, characterized by polar attachment (Banerjee et al., 2015). In this phase, planktonic cells that are freely suspended in the environment recognize a surface to adhere to and begin the attachment process. The bonding of these planktonic cells to the surface is temporary, and the bacterial cell retains its motility structures, such as flagella and pili, throughout this stage (Annous et al., 2009). Subsequently, there is a reorientation of microorganisms to lie flat on surfaces, achieving irreversible attachment and developing resistance to various physical factors that could impede biofilm formation (Banerjee et al., 2015). The irreversible adherence of bacterial cells to the substrate surface becomes evident through the expression of quorum sensing signaling molecules and the creation of extracellular polymeric substance (EPS) (Annous et al., 2009).

Following the effective attachment of microorganisms to surfaces, the adherent microorganisms initiate reproduction and clustering within the extracellular polymeric substance (EPS) they generate, resulting in the formation of micro-colonies (Rabin et al., 2015).

The extracellular polymeric substance (EPS) plays a vital role in the maturation of biofilms by aiding in microbial attachment to surfaces, stabilizing the three-dimensional structure of the biofilm, clustering cells together, and providing protection against various stresses such as the host immune system response, antimicrobials, oxidative damage, and metallic cations. Additionally, EPS encapsulates essential signaling

molecules required for quorum sensing, metabolic products, and enzymes (Toyofuku et al., 2016). A mature biofilm may exhibit a structured "mushroom" or "tower" shape, with microorganisms arranged based on aero-tolerance and metabolism rate (Rabin et al., 2015). A developed biofilm consists of three tiers: an internal regulatory layer, an intermediate microbial basement layer, and an exterior layer hosting planktonic microorganisms ready to exit the biofilm (Zhao et al., 2017).

While dispersal can be triggered by variations in environmental factors such as pH and nutrient levels, other conditions promoting the breakup of biofilms involve external forces like fluid shear and abrasion (Kaplan, 2010). Ultimately, a mature biofilm undergoes active rupture (dependent on motility and EPS degradation) or passive dispersion (influenced by physical factors like liquid flow) to release microorganisms and initiate a new cycle of biofilm formation (McDouglad et al., 2012). Cells in a dispersion phase exhibit morphology closer to that of planktonic cells rather than mature biofilm cells, and this resemblance is essential for their role in initiating the formation of a new biofilm (Annous et al., 2009). Key factors contributing to the dispersion of matured biofilms include overpopulation, intense competition, nutrient scarcity (Rabin et al., 2015) and changes in environmental conditions such as temperature, oxygen levels, and the accumulation of metabolites. Additionally, the upregulation of genes related to cell motility and EPS degradation, coupled with the downregulation of genes involved in polysaccharide and fimbriae synthesis, play a significant role in the dispersion process (McDouglad et al., 2012).

1.5.1 Biofilm formation by pathogenic yeasts of *Candida* spp.

The ability to form biofilms is evident in pathogenic microorganisms within the yeast population, specifically those belonging to the *Candida* genus. In this study, five pathogenic species exhibiting biofilm production were identified: *Candida albicans*, *Candida tropicalis*, *Candida krusei*, *Candida glabrata*, and *Candida parapsilosis*.

Biofilms play a significant role in fostering persistent infections within the human body. This is exemplified by the formation of biofilms by *Candida* species, which contribute to both superficial and systemic fungal infections, particularly in individuals with compromised immune systems (Sims et al., 2005). Managing these infections proves challenging due to the inherent traits of these species, such as resistance to

antifungal medications, the expression of virulence factors, and their capability to form biofilms. Notably, biofilm formation is a common aspect of mucosal infections (Ganguly et al., 2011), often involving interactions with both commensal bacterial flora and host components (Dongari-bagtzoglou et al., 2009).

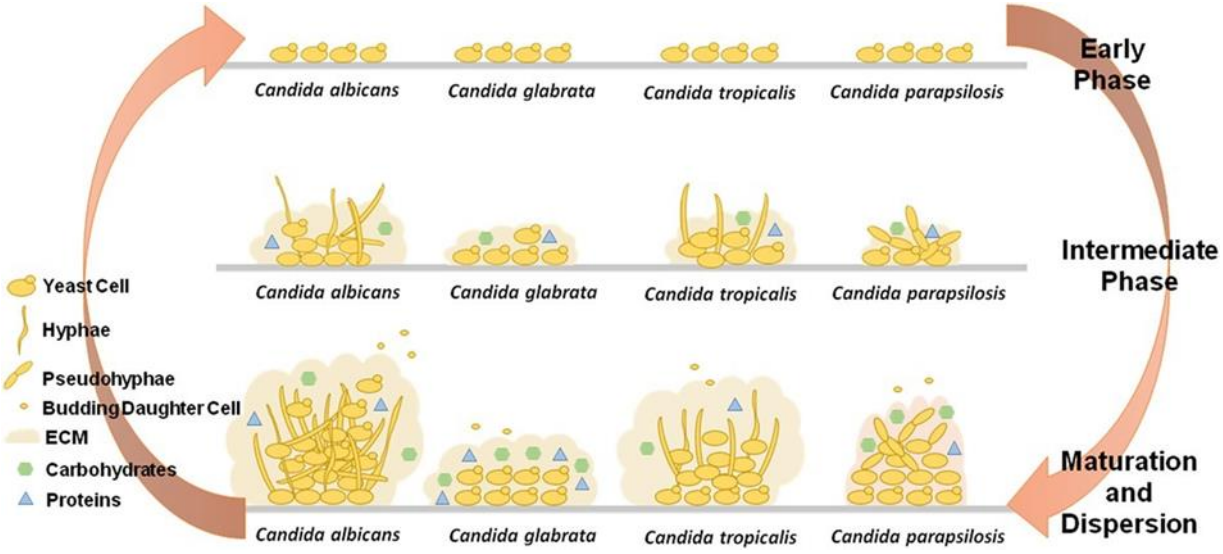

Figure 2. Biofilm formation steps of *Candida* spp. (Cavalheiro et al., 2018).

Various *Candida* species can lead to numerous infections, with *Candida albicans* being the predominant pathogen in *Candida*-related infections, followed by *Candida glabrata* (Tscherner et al., 2011). *Candida tropicalis* is notably associated with urinary tract infections (Rho et al., 2004), while *Candida parapsilosis* is commonly found on the skin of healthy individuals and is a causative agent in catheter-related infections (Yapar et al., 2014). Each *Candida* species exhibits distinct characteristics in terms of biofilm formation, including differences in morphology, extracellular matrix (ECM) composition, and the ability to confer resistance to antifungal agents (Seneviratne et al., 2008). This diversity adds complexity to addressing *Candida* biofilm threats as a unified challenge. Given the increasing prevalence of these fungal infections, there is a pressing need to explore effective therapeutic approaches for more efficient patient treatment. The exploration of different pathogenic features, such as biofilm formation, is essential in the quest for suitable therapeutics.

The process of biofilm formation is present in all the *Candida* species discussed, but it varies significantly depending on factors such as the species itself, the surface

involved, and the host niche. Specifically, mature biofilms of *C. albicans* display a more diverse structure as shown in Figure 2, consisting of blastophores and hyphae enveloped by an extracellular matrix (ECM) made of polysaccharide material (Chandra et al., 2001). This ECM serves as a structural scaffold facilitating cell adhesion to each other and different surfaces, while also acting as a barrier between the biofilm cells and their surrounding environment (Mitchell et al., 2016). Typically, water channels are found surrounding the microcolonies within the biofilm structure (Ramage et al., 2001).

Regarding *C. glabrata*, its biofilm consists solely of yeast form cells arranged in a multilayer structure (Figure 2), densely packed or clustered (Silva et al., 2009). On the other hand, *C. tropicalis* biofilm is characterized by a network comprising yeast, pseudohyphae, and hyphae, featuring pronounced hyphal budding (Bizerra et al., 2008). In contrast, *C. parapsilosis* forms biofilms with clusters of yeast cells adhering to surfaces, characterized by minimal extracellular matrix (ECM) presence (Lattif et al., 2010). These distinctions underscore the intricate nature of the processes governing biofilm formation and the challenge of finding a universal approach for eliminating all *Candida* biofilms. *Candida* biofilms primarily occur in mucosal or endothelial environments, contributing to common candidiasis such as vaginal and oral infections, and are also associated with medical devices like vascular and urinary catheters, as well as dentures (Nett et al., 2016). *C. krusei* is an emerging nosocomial pathogen (Mastromarino et al., 2013), particularly in immunocompromised patients, and it is easily isolated. Unlike other *Candida* species, this organism exhibits a higher temperature optimum of 43-45°C. Despite its lower prevalence compared to species like *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. parapsilosis*, there are significant therapeutic concerns due to its robust resistance to antibiotics (Pfaller et al., 2006).

1.6 Anti-adhesion activity of probiotics against *Candida* species

One of the mechanisms through which probiotics exert their protective effects against *Candida* spp. is through anti-adhesion activity. Probiotics exhibit anti-adhesion activity against *Candida* species through multiple mechanisms, including competition for receptor sites, production of antimicrobial substances, modulation of host immune response, and disruption of biofilm formation. As far as competition for receptor sites

is concerned, LAB can compete with *Candida* species for binding sites on host tissues. LAB adhere to epithelial cells through specific surface adhesins, thereby occupying the receptor sites that *Candida* would otherwise utilize for adhesion (Nobile et al., 2008). By preventing *Candida* adherence to host tissues, LAB effectively inhibit the initial step of *Candida* colonization and subsequent infection. LAB produce various antimicrobial substances such as organic acids, hydrogen peroxide, bacteriocins, and biosurfactants, which can inhibit the growth and adhesion of *Candida* species. For instance, organic acids lower the pH of the surrounding environment, creating an unfavorable condition for *Candida* adhesion and proliferation (Parolin et al., 2015). Hydrogen peroxide exerts direct antifungal activity against *Candida* cells (Osset et al., 2001), while bacteriocins target specific receptors on *Candida* cell surfaces, disrupting adhesion processes (Pérez et al., 2018). LAB have immunomodulatory effects on the host immune system, enhancing the local immune response against *Candida* infections. By stimulating the production of antimicrobial peptides, cytokines, and chemokines, LAB contribute to the reinforcement of epithelial barrier function and the recruitment of immune cells to the site of infection (Salminen et al., 2004). This immune modulation not only helps in controlling *Candida* growth but also aids in the clearance of adherent *Candida* cells from host tissues. *Candida* species often form biofilms on host surfaces, providing protection against host immune defenses and antimicrobial agents. LAB possess the ability to disrupt *Candida* biofilms through various mechanisms. They can produce enzymes such as proteases and glycosidases, which degrade the extracellular matrix of biofilms, weakening their structure (Allonsius et al., 2019). Additionally, LAB-derived biosurfactants interfere with biofilm formation by disrupting microbial cell-cell interactions and inhibiting the adhesion of *Candida* cells to abiotic surfaces (Gago et al., 2011).

LABS from various origins have been investigated for their anti-adhesion properties. Specifically, Zárate and Nader-Macias (2006) observed that *Lactobacillus acidophilus* and *Lactobacillus paracasei*, obtained from the vagina, effectively hindered the attachment of *Staphylococcus aureus* and streptococci. Balcázar et al. (2008) similarly discovered that *Lactococcus lactis*, *Lactiplantibacillus plantarum*, and *Lactobacillus fermentum* could impede the adhesion of various fish pathogens to host intestinal mucus in *in vitro* conditions. Moreover, LAB demonstrate the capability to disrupt the adhesion of pathogens to epithelial cells in the urogenital and intestinal tracts

(Otero et al., 2007). The supernatants produced by LAB contain compounds that reduce the adhesion of pathogenic microorganisms. Numerous LAB are recognized for inhibiting the growth of *Candida* species through mechanisms such as competing for adhesion sites or generating various antagonistic metabolites that impede growth (Rönnqvist et al., 2007).

As already mentioned, *Lactobacillus* species have been recognized for their ability to inhibit pathogens through various mechanisms, including adhesion competition, the production of acids, bacteriocins, biosurfactants, hydrogen peroxide, and coaggregation molecules (Orsi et al., 2014; Sabia et al., 2014). Additionally, they produce molecules with biosurfactant properties that impact the initial adhesion of *C. albicans* to host surfaces and hinder biofilm growth (Ceresa et al., 2015). Furthermore, probiotics can activate the immune system in their host, leading to the production of interleukins. These interleukins play a crucial role in directing immune responses against fungi, enhancing the host's immune system's effectiveness in combating *C. albicans* infections (Li et al., 2019).

As outlined earlier, probiotics exhibit diverse mechanisms of action against *C. albicans*. Given the potential strain-specific nature of these mechanisms, it is important to explore the probiotic attributes of various *Lactobacillus* strains for their potential use in preventing *Candida* infections (Ribeiro et al., 2019).

The initial stage of *C. albicans* pathogenesis involves adherence to the host cell surface, enabling fungal colonization in a specific niche and the initiation of the infection process. Disrupting this process prevents *C. albicans* from adhering or facilitates easy removal, preventing tissue colonization (Simon et al., 2019). Certain *Lactobacillus* strains have been proposed to hinder *Candida* adherence on mucosal surfaces through exclusion, competition for receptor sites, and displacement of adhered yeast cells (Parolin et al., 2015). Verdenelli et al. (2014) discovered anti-adhesive properties in five distinct *Lactobacillus* strains against *C. albicans*. While all studied strains demonstrated the ability to hinder yeast adhesion, the effectiveness varied depending on the specific strain. This variability suggests that microorganisms within the same genus may employ different mechanisms to counteract *C. albicans*. In a study exploring the impact of *Lactobacillus* on *C. albicans* adhesion, Parolin et al. (2015) assessed 13 *Lactobacillus* strains isolated from the vaginal cavity. Three adhesion

mechanisms were investigated (exclusion, competition, and displacement) and the findings indicated that 10 LAB strains could diminish *C. albicans* adhesion through all investigated mechanisms. Among these, *L. crispatus* BC2, *L. gasseri* BC10, and *L. gasseri* BC1 exhibited the most significant inhibitory activity.

The ability of *Lactobacillus* spp. to hinder *Candida* adherence to host tissues has a direct correlation with the cell surface hydrophobicity of LAB. This hydrophobicity significantly influences their adhesion to epithelial tissue, creating a mechanical barrier against *Candida* adhesion (Itapary dos Santos et al., 2019). A study by Aarti et al. (2018) demonstrated that the *L. pentosus* LAP1, isolated from Hentak (a fermented fish in Manipur, India), exhibited notable auto-aggregation and hydrophobicity percentages. Additionally, it displayed significant antifungal activity against *C. albicans*, *C. tropicalis*, and *C. krusei*. These characteristics position this strain as a potential *Candida*te for biotherapeutic products targeting *Candida* infections. However, given its isolation from Hentak, further research is needed to assess the ability of *L. pentosus* strain LAP1 to colonize the host and maintain inhibitory properties against *C. albicans*. It's important to recognize that *in vitro* results may not necessarily be replicated *in vivo* in humans.

1.7 Antimicrobial properties of probiotics against selected pathogens

Numerous probiotics have been found to generate antimicrobial substances, ranging from small molecules to bioactive peptides. They can impede toxin production and disrupt the ability of certain harmful microorganisms to attach directly to the surface cells. The antimicrobial properties of *Lactobacillus* strains are well-documented against various pathogens such as *Escherichia coli*, *Salmonella*, *Listeria,* and *Staphylococcus aureus* (Varma et al., 2011).

Escherichia coli is recognized as a predominant member of the natural flora residing in the human colonic region. While the majority of strains belonging to this species are benign within the intestinal environment, certain variants have acquired virulence factors, enabling them to provoke a spectrum of human ailments (Nataro et al., 1998). Pathogenic *E. coli* strains are implicated in three primary clinical conditions: urinary tract infections, enteric or diarrheal diseases, and meningitis (Kaper et al., 2004). The principal mechanisms through which *E. coli* induces enteric illnesses

involve attachment and colonization of the intestinal mucosa, manipulation of the host cell cytoskeleton or evasion of host immune defenses, and the secretion of toxins (Torres, 2009). Existing approaches to combat pathogenic *E. coli* typically rely on antibiotic usage. However, the emergence of antibiotic-resistant strains poses a significant challenge, as many pathogenic variants have developed resistance to these medications (Collignon, 2009; Tadesse et al., 2012). The escalation of antibiotic resistance has spurred research efforts towards identifying alternative antimicrobial strategies, with probiotics emerging as a promising avenue. The utilization of *Lactobacillus* spp. and *Bifidobacterium* spp. as probiotics for addressing microbial infections and promoting human well-being has served as a catalyst for numerous research endeavors. Studies have demonstrated the antimicrobial properties of various probiotics against pathogens, including *E. coli* (Tejero-Sariñena et al., 2012). Furthermore, investigations have highlighted the ability of probiotics to down-regulate the expression of virulence genes in pathogenic *E. coli* strains, such as *E. coli* O157:H7, through the secretion of bioactive molecules (Medellin-Pena et al., 2007). Additionally, probiotics have been shown to reduce the adhesion of pathogenic *E. coli* strains, including *E. coli* O157: H7 and *E. coli* O127: H6 to epithelial cell monolayers (Erdem et al., 2007). Moreover, the propensity of pathogenic *E. coli* to form biofilms, which contribute to their pathogenicity, has been well-documented (Beloin et al., 2008; Martinez-Medina et al., 2009).

Salmonella is among the most severe pathogens accountable for foodborne diseases, hospitalizations, and fatalities (Liu et al., 2018). *Salmonella* demonstrates a high degree of adaptability within both the natural environment and the host's gastrointestinal tract (Ryan et al., 2015). *Lactobacillus* species have emerged as a primary focus in research aimed at exploring their probiotic properties as a potential alternative for controlling diseases derived from *Salmonella*. Numerous studies have delved into investigating the ability of *Lactobacillus* spp. to combat *Salmonella* infections, both in laboratory settings and in living organisms. There exists a multitude of reports documenting the use of *Lactobacillus* spp. as probiotics to inhibit *Salmonella* growth (Ravaei et al., 2013). For instance, Casey et al. (2007) conducted experiments where pigs were administered with five strains of *Lactobacillus*, leading to a substantial reduction in the fecal count of *Salmonella enterica Serovar* Typhimurium. They further noted a decrease in the incidence, severity, and duration of diarrhea among the treated subjects. In contrast, Voravuthikunchai et al. (2006) observed that *in vitro* investigation

did not reveal any antibacterial effects of *Lactobacillus* spp. isolated from vaginal samples against *Salmonella typhi* and *Salmonella typhimurium*. Pascual et al. (year of publication) demonstrated that oral gavage of *Lactobacillus salivarius* alongside *Salmonella Enteritidis* in chickens resulted in the complete elimination of *Salmonella* presence in the proventriculus after 21 days. Additionally, Nouri et al. reported that *Lactobacillus salivarius* and *Lactobacillus crispatus*, sourced from the chicken gastrointestinal tract, could suppress the growth of *Salmonella* Enteritidis. Conversely, Truusalu et al. (2004) found that oral inoculation of *Salmonella*-infected mice with *Lactobacillus fermentum* and *Lactobacillus acidophilus* did not exhibit any antibacterial effects against *Salmonella typhimurium. Lactiplantibacillus plantarum* is widely recognized as a probiotic species known for its ability to antagonize *Salmonella* spp. Liu et al. (2018) identified that *Lactiplantibacillus plantarum* demonstrated distinct properties in its response to *Salmonella* infection.

The *Listeria* genus comprises Gram-positive, non-spore-forming, facultative anaerobic, rod-shaped bacteria (Orsi et al., 2011). These bacteria found in diverse environmental sources including soil, water, food, and human and animal feces (Zunabovic et al., 2011), exhibit the ability to thrive under various conditions such as low temperatures, high salt concentrations, and a broad pH range (Walker et al., 1990). Among the *Listeria* species, *Listeria monocytogenes* is the most pathogenic, presenting substantial hazards to both public health and food safety. It is known for causing a highly fatal opportunistic foodborne infection termed listeriosis (Vázquez-Boland et al., 2001). LAB have demonstrated effectiveness in suppressing the growth of *L. monocytogenes* in various food items including fresh and cooked meats, vacuum-packaged meat, and cold-smoked fish (Koo et al., 2012). A previous study conducted by Amezquita et al. (2002) investigated the ability of three LAB strains, *Lactobacillus animalis, L. amylovorus*, and *Pediococcus acidilactici* to inhibit *L. monocytogenes* in refrigerated commercial frankfurters, even when the LAB strains faced growth limitations under such conditions. A study by Reza et al. (2019) showed that *L. fermentum* isolated from mouth presented high inhibition activity against *L. monocytogenes*, while *L. paracasei* recorded low inhibition activity against the pathogen. Numerous research investigations highlighted the ability of LAB bacteriocins or enzymes to hinder the growth of *Listeria* in various food categories including fresh and cooked meats, vacuum-packaged meat, and dairy items. These findings indicate that LAB or their metabolic byproducts have the potential to serve as

effective agents in inhibiting *Listeria* activity. The utilization of LAB bacteriocins as bioactive components in food preservation and ensuring food safety emerges as a viable strategy (Yap et al., 2021). Numerous studies have highlighted the effectiveness of bacteriocins in inhibiting *L. monocytogenes*. For instance, rhamnocin from *Lacticaseibacillus rhamnosus* significantly decreased *L. monocytogenes* cell count within 3 hours of exposure (Jeong et al., 2015). Sakacin produced by *L. sakei* was found to disrupt the membrane of *Listeria* cells (Trinetta et al., 2012). Pediocin, derived from *P. acidilactici* led to a remarkable reduction of approximately 5 logs in *Listeria* count within 5 hours (Le Blay et al., 2012). Reuterin produced by *Lactobacillus reuteri*, exhibited potent antilisterial properties (Gao et al., 2019). Nisin demonstrated prolonged suppression of *L. monocytogenes* growth for up to eight weeks under refrigerated conditions and enterocin, employed in salami production, achieved a reduction of 1.67 log cycles in *L. monocytogenes* count (Renye et al., 2009). Furthermore, a study by Guerrieri et al. (2009) revealed that the biofilm formed by *L. plantarum* strains exhibited the ability to impact the survival and proliferation of the pathogen. *L. plantarum* 35d, a bacteriocin producer strain, demonstrated the greatest effectiveness in reducing the presence of *L. monocytogenes* compared to non-producer strains.

Staphylococcus aureus, a Gram-positive bacterium with a spherical shape, is known to continuously inhabit the skin, nostrils, or throat of approximately 25%–30% of the human population. However, it is also responsible for severe infections that can penetrate deeper into the body (Kang et al., 2017). Varma et al. (2010) showed that *Lactobacillus fermentum*, derived from human colonic mucosal biopsy samples, exhibits antimicrobial properties not only against *Staphylococcus aureus* but also against a broader range of enteroinvasive and food-borne pathogens including *Escherichia coli* and *Salmonella*. In the study of Aboulwafa et al. (2017), it was found that two strains of *Lactobacillus*, namely *Lactobacillus rhamnosus* and *Lactobacillus gasseri*, possess antimicrobial and antibiofilm properties. Additionally, they demonstrate inhibitory effects against the proteolytic activity of both *Staphylococcus* aureus and *Escherichia coli*. Based on the data obtained by Soleimani et al. (2010), out of the four LAB tested, *L. acidophilus*, *L. plantarum*, *L. casei*, and *L. reuteri*, *L. plantarum* exhibited the most pronounced inhibitory activity. Subsequently, *Lactiplantibacillus plantarum* and its antimicrobial components could be considered as a viable option for managing *Staphylococcus aureus*. Another research has shown that

Lactobacillus reuteri, obtained from a healthy vaginal environment, and *Lactobacillus fermentum* have notably suppressed methicillin-resistant *S. aureus*. Moreover, studies have indicated that *L. rhamnosus* can displace and eliminate *S. aureus* attached to human intestinal mucus by 39 to 44% (Maxton et al., 2013). Multiple studies have indicated that *Levilactobacillus brevis* possesses notable antimicrobial effects against *Staphylococcus aureus*. Hojjati (2020) highlighted *L. brevis*' capability to compete with, inhibit, and displace *S. aureus* adhesion to host cells. Additionally, Singh et al. (2020) and Chait et al. (2021) provided further evidence of *L. brevis* antimicrobial effectiveness not only against *S. aureus* but also against other harmful bacteria.

2. Purpose of the study

The aim of the present study was to screen and select species belonging to *Lacticaseibacillus rhamnosus*, *Lactobacillus delbrueckii, Levilactobacillus brevis,* and *Lactiplantibacillus plantarum*, isolated from food samples suitable for the formulation of functional foods (food application) or for the development of new feed strategies (feed application) in order to boost human and animal health. In particular, *Lacticaseibacillus rhamnosus* was isolated from donkey milk, *Lactobacillus delbrueckii* from Pecorino and Ragusano cheese, *Levilactobacillus brevis* from sourdough and *Lactiplantibacillus plantarum* from sourdough, donkey milk and olives. In detail, bacteria strains were subjected to: (a) biofilm production ability, (b) anti-adhesion activity against five potential pathogenic *Candida* species, through pre-coating and co-incubation tests, (c) anti-microbial activity against five potential pathogenic *Candida* species, and (d) safety tests including DNase test, Hemolytic test and Gelatinase test.

3. Materials and methods

3.1 Culture conditions of bacteria and yeast strains

Bacteria and *Candida* strains, belonging to the culture collection of the Food Microbiology Laboratory of the Department of Agriculture, Food and Environment (Di3A), University of Catania, were used for the biofilm formation assay, anti-adhesion tests, antimicrobial activity, and safety tests. Thirty-two bacteria strains were assessed for the forenamed activities against *Candida* spp. strains. All the strains have been revived from the glycerol-iced collections in 5 mL of MRSbroth and incubated overnight (16-18 h) at 37 °C. In detail, the selected strains used in this study are reported in Table 2.

Table 2. Bacteria strains used for biofilm formation assay and anti-adhesion tests.

Strains code	Species
Lrh1, Lrh2, Lrh3, Lrh4, Lrh5, Lrh6, Lrh7	*Lacticaseibacillus rhamnosus*
Lbr1, Lbr2, Lbr3, Lbr4, Lbr5, Lbr6, Lbr7, Lbr8, Lbr9, Lbr10	*Levilactobacillus brevis*
Lpl1,Lpl2,Lpl3, Lpl4,Lpl5,Lpl6, Lpl7,Lpl8,Lpl9, Lpl10,Lpl11,	*Lactiplantibacillus plantarum*
Ldb1, Ldb2,Ldb3	*Lactobacillus delbrueckii*
Lbr11	*Levilactobacillus*

3.2 Culture conditions of pathogens

Pathogens, belonging to the culture collection of the Food Microbiology Laboratory of the Department of Agriculture, Food and Environment (Di3A), University of Catania, were used for the anti-adhesion tests, antimicrobial activity. *Candida albicans* ATCC 10231, *C. tropicalis* DSMZ 5991, *C. krusei* DMSZ 70079, *C. glabrata* DMSZ 11226 and *C. parapsilosis* DSMZ 11224 were used as pathogen yeasts for the anti-adhesion tests. Each *Candida* strain, belonging to the Di3A collection was grown in Chromatic *Candida* Agar.

Escherichia coli 105393, *E.coli* 35218, *Listeria monocytogenes*, *Salmonella* and *S. aureus* DSM 1104 were used as target pathogens for the antimicrobial activity. Each pathogen, belonging to the Di3A collection, was grown in different media and incubated for 24 h at 37 °C under constant shaking. In particular, *Listeria*

monocytogenes was grown in *Listeria* Palcam Agar, *S. aureus* in Mannitol Salt Agar (MSA), *Salmonella* in Hektoen Enteric Agar, and *E.coli* strains in Chromatic *E.coli* O157. The target pathogens used in this work are shown in Table 3.

Table 3. Pathogens used for anti-adhesion and antimicrobial tests.

Pathogens	**Activity**
C. albicans ATCC 10231	Anti-adhesion
C. tropicalis DSMZ 5991	Anti-adhesion
C. krusei DMSZ 70079	Anti-adhesion
C. glabrata DMSZ 11226	Anti-adhesion
C. parapsilosis DSMZ 11224	Anti-adhesion
E.coli 105393, *E.coli* 35218	Antimicrobial
Listeria monocytogenes	Antimicrobial
Salmonella	Antimicrobial
S. aureus DSM 1104	Antimicrobial

3.3 Biofilm formation assay

Biofilm formation assay was undertaken in microplates to evaluate the biofilm production of the bacteria strains by using MRS broth medium and MRS broth supplemented with 0.1% Tween 80. A microplate reader was used to adjust the turbidity of bacterial suspensions in order to reach the concentration of 10^9 CFU/mL. Further on, the 96-wells microtiter were filled with 200 µL MRS broth medium and 200 µL MRS broth supplemented with 0.1% Tween 80 in which 20 µL of each strain was inoculated. After 48 h of incubation at 37 °C the wells of microplate were emptied (Figure 3) and gently washed three times with 100 µL of phosphate-buffered saline (PBS) at pH 7.5. Afterwards, a solution of isopropanol:methanol:PBS (1:1:18) and 200 µL of 2% (w/v) crystal violet was added to each well and washed away. After 30 minutes 100 µL of sterile water was added to wash away any excess. Then, the cells were air dried for 3 h and 100 µL of 33% glacial acetic acid (v/v) was added to detach the cells from microplate wells. Ultimately, the microplate was placed in an iMark™ Microplate Absorbance Reader (Biorad) (Figure 4) to measure the optical density (OD) at 595 nm. Ultimately, 100 µL of glacial acetic acid (v/v) was used as a negative control (ODc). The strains were considered as non-biofilm producers ($OD \leq ODc$), weak biofilm producers ($ODc < OD \leq 2 \times ODc$), moderate biofilm producers ($2 \times ODc < OD < 4 \times ODc$),

strong biofilm producers (4×ODc< OD< 8×ODc) and for very strong biofilm producers (8×ODc<OD).

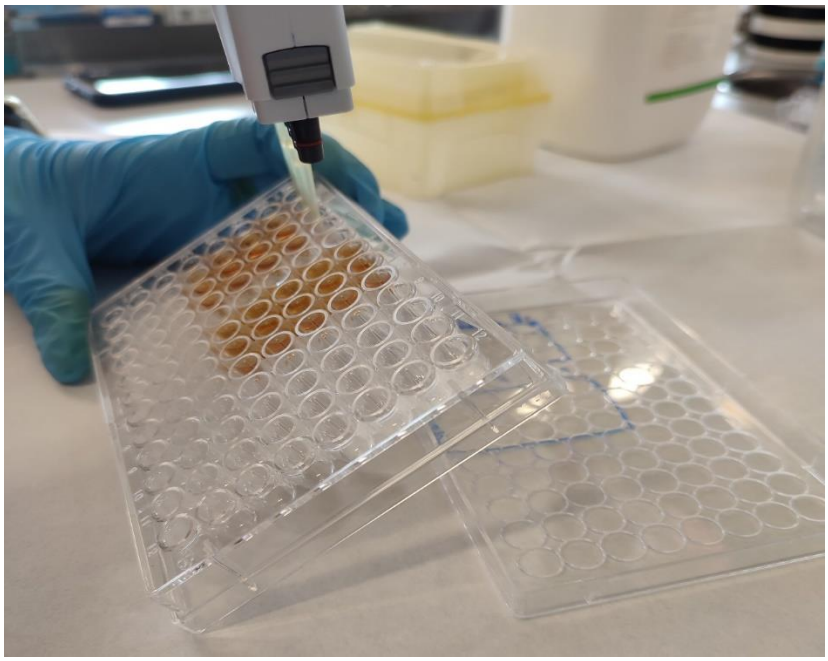

Figure 3. Removal of MRS broth supplemented with 0.1% Tween 80 from the wells by Eppendorf.

Figure 4. iMark™ Microplate Absorbance Reader.

3.4 Anti-adhesion activity against *Candida* spp.

For the anti-adhesion activity against *Candida* spp. suspensions, cell-free supernatant (CFS) of the tested strains were used. CFS of the bacteria strains was obtained by centrifuging 5 mL of the overnight culture of each strain at 11500 rpm for 15 minutes at 4 °C. CFS was then filtered using sterile filters with a pore size of 0.45 µm. The test was performed through two distinct experiments, namely pre-coating and co-incubation.

3.4.1 Pre-coating test

In the pre-coating experiment, 150 µL of the bacteria strains CFS were introduced into a microplate and incubated at 37 °C for 24 h. Afterwards, CFS were removed using a pipette and each well was washed with 100 µL of PBS (pH 7.5) in order to remove the non-adherent cells. Then, 150 µL of 10^7 CFU/mL *Candida* strain suspensions inoculated with strain CFS were added to each well and another 100 µL of *Candida* suspensions without strain CFS were used as control. After 48h of incubation, non-adherent cells were carefully removed by pipette and wells were washed with 100 µL of PBS (pH 7.5). After a while, 100 µL of 99% methanol solution were added and after methanol solution was removed, wells were left to dry for 15 min. Lastly, 100 µL of crystal violet 2% (v/v) were added and then removed using a pipette. Wells were left to dry for 20 min. In the end, wells were filled with 100 µL of glacial acetic acid 33% (v/v) and the microplates were read in a microplate reader at 595 nm. Lastly, the anti-microbial adhesion percentage was calculated using the following equation (Gudina et al., 2010):

$$[\%\text{microbial adhesion} = 1-(OD/OD_c)x100]$$

where, ODc indicates *Candida* spp. suspensions in the CFS whereas OD_0 indicates *Candida* spp. suspensions without CFS (controls).

3.4.2 Co-incubation test

For the co-incubation experiment, 100 μL of 10^7 CFU/mL *Candida* strains were inoculated and 100 μL of *Candida* suspensions without CFS were used as controls. After 24 hours of incubation non adhering cells were removed using a pipette and wells were washed with 100 μL of PBS (pH 7.5). Further on, 99% of methanol solution was added and left to dry for 15 min. After removal of methanol solution, crystal violet 2% was added and then removed using a pipette. Wells were left to dry for 20 min. In the end, wells were filled with 100 μL of glacial acetic acid 33% (v/v) and the microplates were read in a microplate reader at 595 nm. Lastly, the anti-microbial adhesion, with co-incubation test, percentage was calculated using the following the equation (Gudina et al., 2010):

$$[\%\ \text{reduction in biofilm formation} = 1-(OD_c/OD_o) \times 100]$$

where ODc indicates the *Candida* spp. suspensions in the CFSs whereas the OD_o indicates the *Candida* spp. suspensions without CFSs (controls).

3.5 Anti-microbial activity against selected pathogens

The anti-microbial activity of the 32 bacteria strains, both cells and cells free supernatant (CFS), was tested against some of the most common pathogens using the agar well diffusion assay. For the anti-microbial activity of the strains' cells against pathogens, a microplate reader was used to adjust the turbidity of bacterial suspensions in order to reach a population of 10^9 CFU/mL. Further on, the CFS of the strains was obtained by centrifuging 5 mL of the overnight culture of each strain at 11500 rpm for 20 min at 4 °C. The CFS was then filtered using sterile filters with a pore size of 0.22 μm. Afterwards, standardized pathogens were swabbed and once the surface of the agar plate was dried, 20 μL of each probiotic strain was suspended. Pathogen standardization was performed by using McFarland Equivalence Standards in order to obtain a cell density of 10^9 CFU/mL as initial inoculum. After 24 h of incubation at 37 °C, the diameter of inhibition growth zones around the disk was measured.

3.6 Safety tests

The selected strains were subjected to phenotypical assays to characterize their safety properties. DNase, hemolytic and gelatinase tests are essential for ensuring that bacteria strains are non-pathogenic and safe for their intended applications, whether in food production or as probiotics. They help in identifying and excluding any strains that might pose a risk to human health.

3.6.1 DNase test

The production of DNase is associated with many pathogenic bacteria. DNase breaks down DNA, helping pathogens evade the immune system and spread infection. Ensuring bacteria strains do not produce DNase helps confirm their safety. Since the selected strains are used in food products and probiotics, the absence of DNase activity reassures that the strains are non-pathogenic and safe for consumption. A DNase test negative result is indicated by the absence of clear zones around the bacterial colonies. The area surrounding the colonies retains the original color of the medium, indicating that the bacteria do not produce DNase and have not hydrolyzed the DNA in the agar. In contrast, a positive DNase test result is indicated by the presence of clear zones around the bacterial colonies, signifying that the bacteria have produced DNase and hydrolyzed the DNA in the agar. For the DNase test, all strains were revived from the glycerol-iced stock cultures in 5 mL of MRS broth and incubated overnight at 37 °C. After 24 h of incubation, a drop of 5 µL of each strain was placed on the DNA agar media plate.

3.6.2 Hemolytic test

Hemolysis refers to the breakdown of red blood cells. Pathogenic bacteria often exhibit hemolytic activity, which can damage host tissues and contribute to disease. Bacteria strains used in food and probiotics should not exhibit hemolytic activity. Testing for hemolysis ensures that the strains are not harmful and are safe for human consumption. The absence of hemolytic activity indicates that the strains lack this particular virulence factor, reinforcing their suitability for safe use. For the hemolytic

test, revitalized bacteria strains were streaked on trypticase soya agar (Oxoid) with 5% (v/v) defibrinated sheep blood. After 24 h of incubation at 37 °C, the hemolytic activity of each strain was observed and classified as total or β-hemolysis (clear halos around the colonies), partial or α-hemolysis (greenish halos around the colonies), or γ-hemolysis (absence of hemolysis). *Proteus mirabilis* was used as positive control (Figure 5).

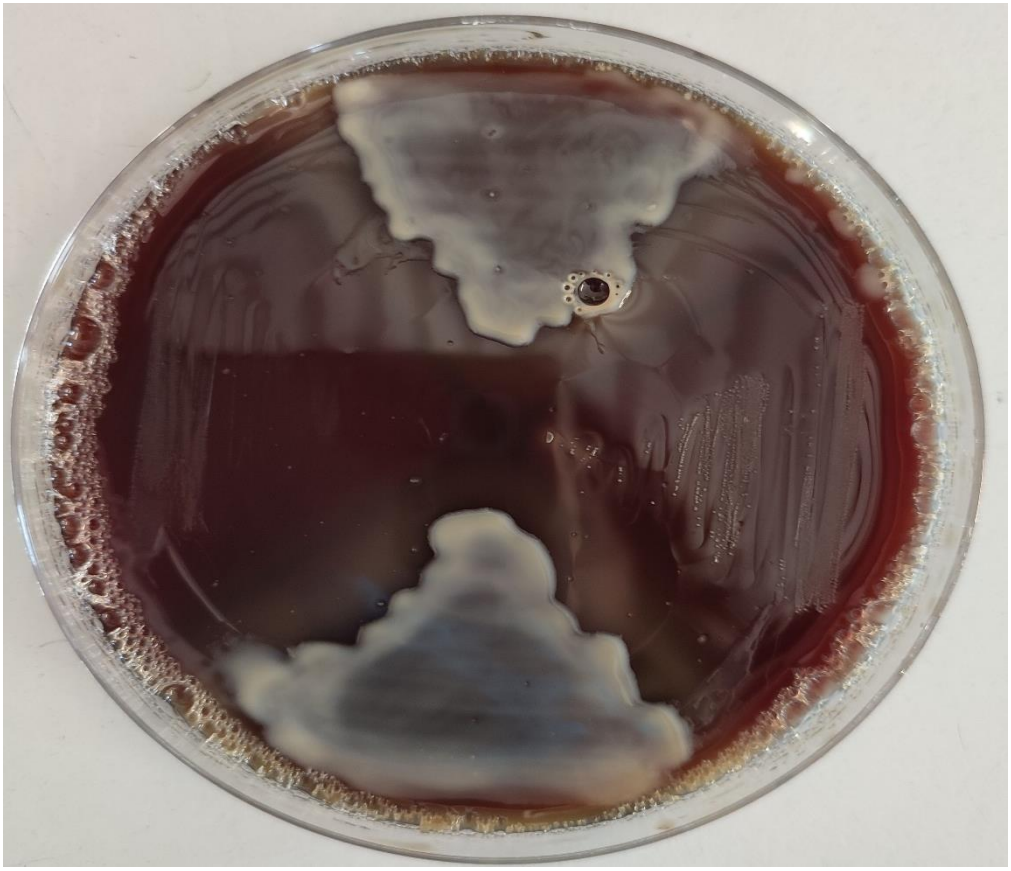

Figure 5. Proteus mirabilis used as positive control in hemolytic test.

3.4.1 Gelatinase test

Gelatin, a protein obtained from the connective tissues of vertebrates, originates from collagen and it is formed when collagen is boiled in water. The process of gelatin hydrolysis is used to identify the presence of gelatinases. Gelatinases are enzymes secreted by some bacteria that break down or digest gelatin. Gelatinase is a key enzyme in many pathogenic organisms can act as virulence factors by dissolving the host's connective tissues, facilitating invasive infections. Gelatin hydrolysis is indicated by

clear zones around colonies that are gelatinase-positive. In contrast, negative gelatin hydrolysis is indicated by the lack of a clear zone around the colony. This test identifies whether bacteria can produce gelatinases (Leboffe et al., 2010). For the gelatinase test, all strains have been revitalized from the glycerol-iced collections in 5 mL of MRS broth and incubated overnight at 37 °C. After 24 h of incubation, a drop of 5 µL of each strain was placed on a Gelatinase agar media plate.

4. Results and Discussion

4.1 Biofilm formation assay

The results of the biofilm formation assay of 32 bacteria strains in MRS broth and MRS supplemented with Tween 80 are shown in Table 4. The capability of the strains to form biofilm was categorized in the following classes: non-biofilm producers (OD≤ODc), weak biofilm producers (ODc<OD≤2×ODc), moderate biofilm producers (2×ODc<OD<4×ODc), strong biofilm producers (4×ODc<OD<8×ODc), and very strong biofilm producers (8×ODc<OD). The term ODc corresponds to optical density values of 0.34 and 0.36 obtained from MRS broth and MRS supplemented with Tween 80, respectively, without cell suspension.

According to the results and ODc values obtained, the method applied has shown a lot of variability between the strains. Specifically, by using MRS broth medium, 5 strains were very strong biofilm producers, 2 belonging to *L. brevis* (Lbr6, Lbr10) and 3 belonging to *L. rhamnosus* (Lrh1, Lrh2 Lrh7). Sixteen out of thirty-two strains were strong biofilm producers, namely 5 strains belonging to *L. brevis* (Lbr1, Lbr2, Lbr3, Lbr4, Lbr5), 7 strains belonging to *L. plantarum* (Lpl1, Lpl2, Lpl3, Lpl4, Lpl5, Lpl7, Lpl8,), 3 strains belonging to *L. rhamnosus* (Lrh3, Lrh4, Lrh5), and 1 strain belonging to *L. delbrueckii* (Ldb1). One strain belonging to *L. rhamnosus (Lrh6)* was moderate biofilm producer and the remaining 8 strains were weak biofilm producers, With 4 belonging to *L. brevis* (Lbr7, Lbr8, Lbr9, Lbr11), 2 to *L. delbrueckii* (Ldb2, Ldb3), and 2 to *L. plantarum* (Lpl6, Lpl10).

Biofilm production of bacteria strains by using MRS broth supplemented with 0.1% Tween 80 presented minor differences compared to using only MRS broth. In particular, 6 strains, 3 belonging to *L. brevis* (Lbr2, Lbr6, Lbr10) and 3 to *L. rhamnosus* (Lrh1, Lrh5, Lrh7), were very strong biofilm producers. Further on, 16 strains, 8 belonging to *L. plantarum* (Lpl1, Lpl2, Lpl3, Lpl4, Lpl5, Lpl7, Lpl8, Lpl9), 4 to *L. brevis* (Lbr1, Lbr3, Lbr4, Lbr5), 3 to *L. rhamnosus* (Lrh2, Lrh3, Lrh4), and 1 to *L. delbrueckii* (Ldb1) were strong biofilm producers. Five strains, 2 belonging to *L. brevis* (Lbr7, Lbr8), 2 to *L. plantarum* (Lpl6, Lpl11), and 1 to *L. rhamnosus* (Lrh6) were moderate biofilm producers. Four strains, 2 belonging to *L. brevis* (Lbr9, Lbr11), 1 to *L. delbrueckii* (Ldb3), and 1 to *L. plantarum* (Lpl6, Lpl10) were weak biofilm

producers. Finally, 1 strain belonging to *L. delbrueckii* (Ldb2) did not present any biofilm production.

Table 4. Biofilm production values in MRS broth and MRS supplemented with Tween 80, expressed as optical density values at 650 nm (OD_{650}). Data are average values of three replications.

	Optical Density			
	MRS	**Type of production**	**MRS+Tween80**	**Type of production**
Lbr1	1.857	strong	1.682	strong
Lbr2	2.216	strong	2.832	very strong
Lbr3	1.725	strong	1.742	strong
Lbr4	2.286	strong	1.991	strong
Lbr5	2.570	strong	2.868	strong
Lbr6	2.910	very strong	2.907	very strong
Lbr7	0.567	weak	0.713	moderate
Lbr8	0.615	weak	0.686	moderate
Lbr9	0.457	weak	0.479	weak
Lbr10	4.500	very strong	4.500	very strong
Lbr11	0.392	weak	0.433	weak
Ldb1	1.463	strong	1.807	strong
Ldb2	0.512	weak	0.335	non
Ldb3	0.400	weak	0.432	weak
Lpl1	1.575	strong	1.643	strong
Lpl2	1.323	strong	1.880	strong
Lpl3	1.339	strong	1.698	strong
Lpl4	2.115	strong	2.120	strong
Lpl5	1.061	strong	1.886	strong
Lpl6	0.521	weak	0.947	moderate
Lpl7	1.671	strong	1.731	strong
Lpl8	1.428	strong	1.656	strong
Lpl9	0.958	moderate	1.355	strong
Lpl10	0.394	weak	0.383	weak
Lpl11	0.811	moderate	1.008	moderate
Lrh1	3.069	very strong	3.099	very strong
Lrh2	2.926	very strong	2.399	very strong
Lrh3	1.688	strong	1.860	strong
Lrh4	2.282	strong	2.278	strong
Lrh5	1.603	strong	2,728	very strong
Lrh6	0.759	moderate	1.163	moderate
Lrh7	3.401	very strong	3.201	very strong

4.2 Anti-adhesion activity against *Candida* species

4.2.1 Pre-coating

The results of the cell-free supernatants (CFSs) of the 32 bacteria strains exhibiting anti-adhesion activity against 5 *Candida* species in pre-coating activity are reported in Table 5. It was shown that the supernatant (CFS) of strain Lbr1 presented the highest inhibition activity against *C. krusei* (62.2%) and *C. tropicalis* (53.3%), while against *C. glabrata* (42.3%), *C. parapsilosis* (30.8%), and *C. albicans* (22.8%) Lbr1 presented lower inhibition activity. The CFS from strain Lbr2 exhibited the highest anti-adhesion activity against *C. glabrata* (38.6%) and *C. parapsilosis* (37.9%). However, as Table 5 shows, a lower activity level against *C. albicans* (22.4%), *C. tropicalis* (29.8%), and *C. krusei* (27.7%) was observed for this strain. The anti-adhesion activity of the CFS from Lbr3 has demonstrated a lower microbial adhesion against *C. albicans* (21.4 %). However, the anti-adhesion activity of Lbr3 was higher against *C. tropicalis* (39.5%), *C. krusei* (44.4%) and *C. parapsilosis* (38.8%), but the best performance of this strain was observed against *C. glabrata* (67.3%). The CFS from Lbr4 strain exhibited its highest activity against *C. parapsilosis* (47.5%), while the lower activity was expressed against *C. albicans* and *C. krusei*, 16.4% and 13.9%, respectively, whereas inhibition against *C. tropicalis* (23.8%) and *C. glabrata* (21.4%) was also low. Further on, Table 5 reveals that the CFS from Lbr5 strain presented a moderate anti-adhesion activity, showing a percentage of 48.7% against *C. glabrata*, followed by *C. parapsilosis*, *C. albicans*, and *C. tropicalis*, with 41.7%, 38.5%, and 33.2% respectively. However, the lowest anti-adhesion activity was found against *C. krusei* (19.6%). Lbr6 strain exhibited low inhibition against *C. albicans*, *C. tropicalis*, *C. krusei*, and *C. glabrata* with percentages below 15%, Notably, this strain showed higher, inhibition against *C. parapsilosis*, with a percentage of 46.7%.

Finally, the CFS from Lbr7 strain has also shown moderate anti-adhesion activity against *C. tropicalis* (44.5%), *C. glabrata* (41.0%), and *C. parapsilosis* (34.1%). Inside of this species, the CFS of Lbr8 strain has demonstrated the lowest results in the pre-coating experiments. The only highest value was obtained against *C. parapsilosis* (46.7%). CFS of Lbr9 strain has also demonstrated one of the lowest results in the pre-coating experiments inside of this species. Highest inhibition was recorded against *C. krusei* (34.8%). The percentage values revealed by testing CFS of strain Lbr10

demonstrated that *C. krusei* showed the highest susceptibility to inhibition with a mean of 44.0%, followed closely by *C. albicans* at 39.0%. For Lbr11's CFS, the strain showed significant inhibitory activity against various *Candida* species. The highest susceptibility was observed in *C. tropicalis* and *C. parapsilosis*, with a mean inhibition of 71.5% and 65.9% respectively. *C. albicans* also exhibited substantial inhibition at 53.5%. Additionally, notable inhibition was observed against *C. krusei* (46.9%) and *C. glabrata* (42.9%).

Ldb1's strain exhibited moderate inhibitory activity against *C. krusei* (46.7%) and *C. glabrata* (42.2%), with lower inhibition against *C. parapsilosis* (32.5%). Ldb2's strain demonstrated moderate inhibition, *C. tropicalis* showing the highest susceptibility (37.2%) followed by *C. krusei* (35.5%) and C. glabrata (26.0%). Ldb3's strain showed great inhibition (60.5%) against *C. tropicalis*, while demonstrating moderate inhibition percentage of 35.6% and 36.3% against *C. krusei* and *C. glabrata*, respectively. Lpl1's strain exhibited low values against all *Candida* species. CFS of Lpl2 strain also exhibited low inhibition with a slightly higher inhibition observed against *C. glabrata* at 20.1% and *C. parapsilosis* at 33.2%. Inside of this species, the CFS of Lpl3 strain has demonstrated the lowest results in the pre-coating experiments with percentages ranging under 23%. CFS of Lpl4 strain also showed low inhibtion rate with performance value against *C. krusei* 34.5%. Lpl5's CFS demonstrated low inhibition with percentages around 20-25%. Lpl6's CFS also demonstrated low inhibition activity ranging from 8% to 25% for all *Candida* species. CFS of Lpl7 strain has demonstrated one of the lowest results in the pre-coating experiments inside of this species. The only highest value was obtained against *C. parapsilosis* (41.1%).

According to Table 5, low inhibition activity was also reported for CFS of Lpl8. Lpl9's CFS strain has reported a moderate anti-adhesion activity, showing a percentage value of 51.9% against *C. glabrata*, followed by 43.3%, 39.8% and 33.4% against *C. parapsilosis*, *C. krusei* and *C. tropicalis* respectively. The CFS of Lpl10 has shown appreciable percentage values of anti-adhesion activity against *C. albicans* (75.0%) and *C. tropicalis* (68.6%) and moderate against *C. parapsilosis* (34.1%). The CFS of Lpl11 strain also showed high values of anti-adhesion activity against *C. albicans* (70.1%) and C. tropicalis (68.1%). In contrast the values expressed against *C. krusei* and *C. glabrata* were relatively moderate with the values of 48.3% and 37.7% respectively.

Table 5. Anti-adhesion values of the pre-coating test expressed as percentage of microbial adhesion by bacteria strains' CFS against five pathogenic *Candida* spp. Data are average values of three replications ± standard deviation.

	C. albicans	*C. tropicalis*	*C. krusei*	*C. glabrata*	*C. parapsilosis*
Lbr1	22.8±0.05	53.3±0.01	62.2±0.03	42.3±0.04	30.8±0.1
Lbr2	22.4±0.09	29.8±0.07	27.7±0.02	38.6±0.06	37.9±0.06
Lbr3	21.4±0.03	39.5±0.03	44.4±0.05	67.3±0.06	38.8±0.09
Lbr4	16.4±0.02	23.8±0.07	13.9±0.10	21.4±0.01	47.5±0.04
Lbr5	38.5±0.04	33.2±0.08	19.6±0.04	48.7±0.04	41.7±0.01
Lbr6	5.6±0.10	4.9±0.07	7.4±0.28	10.5±0.18	46.7±0.12
Lbr7	23.3±0.09	44.5±0.10	11.0±0.06	41.0±0.09	34.1±0.10
Lbr8	5.6±0.10	4.9±0.07	7.4±0.28	10.5±0.18	46.7±0.12
Lbr9	5.1±0.05	no inhibition	34.8±0.06	14.4±0.06	27.2±0.05
Lbr10	39.0±0.24	8.2±0.16	44.0±0.24	13.1±0.23	no inhibition
Lbr11	53.5±0.22	71.5± 0.30	46.9± 0.06	42.9±0.13	65.9± 0.07
Ldb1	no inhibition	no inhibition	46.7±0.04	42.2±0.03	32.5±0.03
Ldb2	16.1±0.13	37.20±0.13	35.5±0.14	26.0±0.17	no inhibition
Ldb3	1.0± 0.09	60.5± 0.24	35.6± 0.14	36.3± 0.30	no inhibition
Lpl1	16.1±0.06	3.6±0.06	19.9±0.05	7.9±0.03	6.8±0.02
Lpl2	6.2±0.05	8.1±0.06	10.2±0.14	20.1±0.10	33.2±0.18
Lpl3	no inhibition	7.8±0.08	0.9±0.01	5.6±0.09	22.5±0.22
Lpl4	15.8±0.05	18.2±0.2	34.5±0.05	19.0±0.05	14.3±0.08
Lpl5	no inhibition	20.9±0.20	21.1±0.19	no inhibition	24.2±0.17
Lpl6	no inhibition	14.5±0.02	24.7±0.08	8.1±0.02	11±0.11
Lpl7	no inhibition	no inhibition	no inhibition	16.2±0.05	41.1±0.11
Lpl8	no inhibition	9.9±0.02	5.0±0.10	no inhibition	28.5±0.10
Lpl9	no inhibition	33.4±0.03	39.8±0.06	51.9±0.16	43.3±0.10
Lpl10	75.0±0.05	68.6±0.34	no inhibition	18.8±0.35	34.1±0.18
Lpl11	70.1±0.02	68.1±0.35	48.3±0.05	37.7±0.09	no inhibition
Lrh1	no inhibition	37.2±0.04	56.6±0.07	33.4±0.06	50.1±0.07
Lrh2	no inhibition	no inhibition	16.5±0.11	38.4±0.18	44.2±0.15
Lrh3	no inhibition	42.7±0.03	58.8±0.02	27.8±0.03	53.4±0.08
Lrh4	no inhibition	13.0±0.30	27.8±0.15	no inhibition	38.5±0.16
Lrh5	4.6±0.04	13.2±0.11	1.4±0.06	25.6±0.09	no inhibition
Lrh6	no inhibition	35.7±0.08	50.4±0.02	26.3±0.06	51.8±0.07
Lrh7	28.1±0.16	30.4±0.10	50.8±0.12	28.7±0.17	8.9±0.05

Lrh1 strain displayed moderate to high inhibition against the other species tested, with percentages ranging from 33.4% for *C. glabrata* to 56.6% for *C. krusei*. Notably, it exhibited the highest inhibition against *C. krusei*, followed by *C. parapsilosis* (50.1%), suggesting pronounced effectiveness against these species compared to *C. tropicalis* (37.2%) and *C. glabrata*. Percentages of Lrh2 strain revealed low levels of effectiveness in inhibiting *Candida* species. Higher inhibition against *C. glabrata* (38.4%) and *C. parapsilosis* (44.2%) was observed. The CFS of strain Lrh3 did not exhibit any activity against *C. albicans*. However, the strain revealed moderate to high inhibition against the other species assayed. Specifically, it displayed the highest inhibition against *C. krusei* (58.8%) and *C. parapsilopsis* (53.4%), followed by *C. tropicalis* (42.7%) and *C. glabrata* (27.8%). As Table 5 reveals, the highest inhibition of CFS's strain Lrh4 was observed for *C. parapsilosis* (38.5%), followed by *C. krusei* (27.8%) and *C. tropicalis* (13.0%). The CFS from strain Lrh5 exhibited low inhibition against *C. albicans*, *C. tropicalis*, *C. krusei,* and *C. glabrata* (< 26%). Additionally, no inhibition was detected against *C. parapsilosis*. There was no inhibition observed against *C. albicans* for Lrh6's CFS. However, the strain demonstrated relatively moderate inhibition against *C. glabrata* (26.3%) and *C. tropicalis* (35.7%), while showing high inhibition percentages against *C. krusei* (50.4%) and *C. parapsilosis* (51.8%). High inhibition against *C. krusei* (50.8%) was observed using the CFS from the strain Lrh7, followed by *C. tropicalis* (30.4%), *C. glabrata* (28.7%), and *C. albicans* (28.1%), whereas the lowest inhibition level was recorded for *C. parapsilosis* (8.9%).

4.2.2 Co-incubation

The results of the cell-free supernatants (CFSs) of the 32 strains which have shown anti-adhesion activity against 5 *Candida* species in co-incubation activity are reported in Table 6.

Table 6. Anti-adhesion values of the co-incubation test expressed as percentage of reduction in biofilm formation against *Candida* spp. Data are average values of three replications ± standard deviation.

	C. albicans	*C. tropicalis*	*C. krusei*	*C. glabrata*	*C. parapsilosis*
Lbr1	30.3±0.10	73.6±0.02	85.3±0.01	6.2±0.02	71.9±0.01
Lbr2	35.4±0.08	46.7±0.06	43.4±0.05	36.4±0.12	56.4±0.05
Lbr3	77.2±0.01	83.0±0.01	39.3±0.02	39.0±0.06	29.4±0.16
Lbr4	38.5±0.07	37.1±0.12	58.6±0.04	39.8±0.02	66.5±0.11
Lbr5	54.4±0.04	19.8±0.05	61.3±0.06	58.0±0.04	76.3±0.12
Lbr6	11.6±0.14	6±0.14	37.8±0.10	no inhibition	38.4±0.02
Lbr7	20.5±0.16	38.2±0.13	42.7±0.04	32.9±0.05	49.4±0.03
Lbr8	11.6±0.14	6.0±0.14	37.8±0.10	no inhibition	38.4±0.02
Lbr9	0.5±0.09	11.9±0.29	19.4±0.29	5.9±0.05	4.3±0.34
Lbr10	64.8±0.02	54.1±0.05	39.7±0.13	17.8±0.16	no inhibition
Lbr11	65.0±0.15	48.8± 0.20	49.1± 0.10	47.7± 0.13	35.1± 0.18
Ldb1	9.6±0.04	no inhibition	36.2±0.44	43.5±0.11	28.1±0.12
Ldb2	55.8±0.05	32.2±0.08	43.6±0.07	45.4±0.12	61.9±0.1
Ldb3	64.8±0.01	7.8±0.27	67.4±0.01	no inhibition	74.9±0.03
Lpl1	18.4±0.16	18±0.07	30.7±0.12	24.7±0.06	18±0.01
Lpl2	7.5±0.08	29.3±0.16	28.3±0.36	no inhibition	21.3±0.13
Lpl3	1.0±0.04	11.5±0.16	36.4±0.22	6.5±0.07	no inhibition
Lpl4	25.4±0.01	5.6±0.07	61.3±0.00	42.4±0.02	24.7±0.04
Lpl5	28.6±0.13	33.2±0.08	33.6±0.08	no inhibition	29.3±0.13
Lpl6	58.8±0.31	31±0.47	33±0.43	45.6±0.42	36.9±0.40
Lpl7	17.5±0.16	no inhibition	0.5±0.10	46.1±0.06	48.3±0.07
Lpl8	33.5±0.02	9.9±0.01	19.9±0.12	43.5±0.12	10.4±0.02
Lpl9	6.3±0.10	19.3±0.11	19.2±0.02	12.7±0.06	46.4±0.07
Lpl10	59.9±0.02	61.5±0.11	39.0±0.17	54.2±0.05	55.8±0.07
Lpl11	74.5±0.05	63.4±0.09	34.0±0.12	65.9±0.08	45.7±0.08
Lrh1	31.4±0.06	30.3±0.01	37.2±0.25	45.2±0.01	31.2±0.13
Lrh2	0.5±0.21	no inhibition	no inhibition	28.8±0.16	5.7±0.07
Lrh3	41.8±0.05	no inhibition	22.0±0.06	26.9±0.08	15.6±0.06
Lrh4	no inhibition	13.0±0.30	27.8±0.15	no inhibition	38.5±0.16
Lrh5	4.6±0.04	13.2±0.11	1.4±0.06	25.6±0.09	no inhibition
Lrh6	54.3±0.05	27.0±0.11	27.9±0.04	33.7±0.07	28.2±0.05
Lrh7	19.0±0.16	37.1±0.20	28.8±0.27	36.8±0.21	no inhibition

The results of the anti-adhesion activity in the co-incubation test of the selected strains against biofilm formation from Candida spp. are summarized in Table 6. The CFS of *L. brevis* Lbr1 presented spp. the highest anti-adhesion activity for *C. krusei* (85.3%) followed by *C. tropicalis* and *C. parapsilosis* with 73.6% and 71.9%, respectively, while it exhibited moderate anti-adhesion activity against *C. albicans* (30.3%). Lbr2 strain exhibited moderate inhibition against *C. albicans* (35.4%), *C. krusei* (43.4%), *C. glabrata* (36.4%) and *C. tropicalis* (46.7%). Additionally, it showed high inhibition percentage against *C. parapsilosis* (56.4%). Notably, CFS of Lbr3 exhibited great inhibition against both *C. albicans* (77.2%) and *C. tropicalis* (83.0%). However, it displayed lower inhibition against *C. krusei* (39.3%), *C. glabrata* (39.0%), and *C. parapsilosis* (29.4%). Lbr4's CFS showed moderate inhibition against *C. albicans* (38.5%), *C. tropicalis* (37.1%) and *C. glabrata* (39.8%). However, it demonstrated high inhibition against *C. krusei* (58.6%) and the highest inhibition against *C. parapsilosis* (66.5%). One of the greatest results were observed in strain Lbr5 since almost all *Candida* spp. demonstrated values higher than 54% (Table 6). In particular, it displayed the highest inhibition against C. *parapsilosis* (76.3%), while against *C. albicans*, *C. krusei*, and *C. glabrata* showing also really high inhibition with percentages of 54.4%, 61.3% and 58.0% respectively. However, inhibition against *C. tropicalis* was lower compared to the other species tested (19.8%). The strain Lbr6 exhibited low to moderate inhibition against C. albicans (11.6%), *C. tropicalis* (6%), *C. krusei* (37.8%) and *C. parapsilosis* (38.4%). However, it showed no inhibition against *C. glabrata*. The CFS of Lbr7 presented moderate inhibition against most species, with percentages ranging from 20.5% for *C. albicans* to 49.4% for *C. parapsilosis*. Notably, it showed slightly higher inhibition against *C. krusei* (42.7%) and *C. parapsilosis* compared to *C. tropicalis* (38.2%) and *C. glabrata* (32.9%). As depicted in Table 6, Lbr8's CFS illustrated low to moderate inhibition against C. tropicalis (6.0%), *C. albicans* (11.6%), *C. krusei* (37.8%) and *C. parapsilosis* (38.4%) while it showed no inhibition against *C. glabrata*. One of the lowest values were observed in strain Lbr9 since percentages of all *Candida* spp. were under 20%. In detail, 0.5% inhibition rate was demonstrated for *C. albicans*, 4.3% for *C. parapsilosis*, 5.9% for *C. glabrata*, 11.9% for *C. tropicalis* and 19.4% for *C. krusei*. Percentages of Lbr10 strain exposed really high inhibition against *C. albicans* (64.8%) and *C. tropicalis* (54.1%). However, it showed lower inhibition against *C. krusei* (39.7%) and *C. glabrata* (17.8%) and no inhibition against *C. parapsilosis*. CFS of Lbr11 revealed

moderate inhibition against most species, with percentages ranging from 35.1% for *C. parapsilosis* to 49.1% for *C. krusei*, with *C. tropicalis* and *C. glabrata* in-between. Notably, as illustrated in Table 6, it showed great inhibition against *C. albicans* (65.0%) compared to the other species tested.

Ldb1 strain exhibited low inhibition against *C. albicans* (9.6%), and *C. parapsilosis* (28.1%) and moderate inhibition against *C. krusei* (36.2%), *C. glabrata* (43.5%). However, it showed no inhibition against *C. tropicalis*. Ldb2 strain displayed moderate to high inhibition, with percentages ranging from 32.2% for *C. tropicalis* to 61.9% for *C. parapsilosis*. Notably, it exhibited the highest inhibition against *C. parapsilosis* , followed by *C. albicans* (55.8%), suggesting pronounced effectiveness against these species compared to *C. tropicalis* (32.2%), *C. krusei* (43.6%), and *C. glabrata* (45.4%). While no inhibition was observed against *C. glabrata* and low against *C. tropicalis* (7.8%) , CFS of Ldb3 revealed impressive results against *C. parapsilosis*, *C. krusei* and *C. albicans* with inhibition of 74.9%, 67.4% and 64.8% respectively (Table 6).

Low inhibition activity was observed by Lpl1 strain against most species, with percentages ranging from 18.0% to 30.7%. Notably, it displayed slightly higher inhibition against *C. krusei* (30.7%) compared to the other species tested. The inhibition percentages against *C. albicans*, *C. tropicalis*, and *C. parapsilosis* were relatively similar, around 18% while against *C. glabrata* around 25%. Lpl2's CFS exhibited low inhibition against *C. albicans* (7.5%) and moderate inhibition against *C. tropicalis* (29.3%), *C. krusei* (28.3%), and *C. parapsilosis* (21.3%). However, it showed no inhibition against *C. glabrata*. Percentages of Lpl3 strain indicated extremely low inhibition against *C. albicans* (1.0%), *C. glabrata* (6.5%) and *C. tropicalis* (11.5%) while it didn't have any inhibition against *C. parapsilosis*. Relatively moderate inhibition was observed against *C. krusei* (36.4%). Lpl4 revealed its best anti-adhesion activity, with high percentage, against *C. krusei* (61.3%). However, a lower activity against *C. glabrata* (42.4%), *C. albicans* (25.4%), *C. parapsilosis* (24.7%) and *C. tropicalis* (5.6%) was found for this strain. CFS of Lpl5 exhibited moderate inhibition against *C. albicans* (28.6%), *C. tropicalis* (33.2%), and *C. krusei* (33.6%), and *C. parapsilosis* (29.3%). However, it showed no inhibition against *C. glabrata*. Lpl6's CFS showed high inhibition against *C. albicans* (58.8%) and moderate inhibition against *C. glabrata* (45.6%) and *C. parapsilosis* (36.9%), *C. tropicalis* (31.0%) and *C.*

krusei (33.0%). Lpl7 strain exposed low values against *C. albicans* (17.5%) and *C. krusei* (0.5%), while there was no inhibition observed against *C. tropicalis*. Furthermore, Table 6 reveals that this strain showed moderate inhibition against *C. glabrata* (46.1%) and *C. parapsilosis* (48.3%). Low inhibition activity was observed by Lpl8 strain against *C. tropicalis, C. parapsilosis* and *C. krusei* with percentages 9.9%,10.4%, 19.9% respectively while moderate inhibition activity was observed against *C. albicans* (33.5%) and *C. glabrata* (43.5%). CFS of Lpl9 exhibited low inhibition against *C. albicans* (6.3%), *C. glabrata* (12.7%), *C. tropicalis* (19.3%) and *C. krusei* (19.2%) except against *C. parapsilosis* whose value was moderate (46.4%). Lpl10 strain revealed great results to almost all *Candida* species. In detail, high inhibition activity was observed against *C. tropicalis* (61.5%), *C. albicans* (59.9%), *C. parapsilosis* (55.8%) and *C. glabrata* (54.2%) and relatively lower inhibition against *C. krusei* (39.0%). Lpl11 also showed good results for nearly every *Candida* species. Notably, it exhibited high inhibition against *C. albicans* (74.5%), *C. glabrata* (65.9%) and *C. tropicalis* (63.4%) while moderate activity against *C. parapsilosis* (45.7%) and *C. krusei* (34.0%).

Lrh1's CFS showed moderate inhibition against all *Candida* species with *C. albicans* at 31.4%, C. tropicalis at 30.3%, C. parapsilosis at 31.2%, C. krusei at 37.2% and *C. glabrata* at 45.0%. In analyzing the inhibition percentages for Lrh2's strain, minimal inhibition against *C. albicans* (0.5%), *C. parapsilosis* (5.7%) and *C. glabrata* (28.0%) was observed , while displaying no inhibitory activity against *C. tropicalis* and *C. krusei*. According to Table 6, CFS of Lrh3 strain has shown its best performance value against *C. albicans* (41.8%), while the lowest was expressed against *C. parapsilosis* with percentage value of 15.6%. Inhibition activity against *C. krusei* (22.0%). and *C. glabrata* (26.9%) was also low and absence of activity effect was observed against *C. tropicalis*. The CFS of Lrh4 strain exhibited no inhibitory activity against both *C. albicans* and *C. glabrata* and low inhibition against *C. tropicalis* (13.0%). Moderate inhibition was observed against *C. krusei* (27.8%) and *C. parapsilosis* (38.5%). Lrh5 strain exhibited really low inhibition against C. albicans and C. tropicalis, *C. krusei* and *C. glabrata* with percentages below 26%, while there was no any inhibition against *C. parapsilosis*. Lrh6's CFS displayed high inhibition against *C. albicans* (54.0%), moderate inhibition against *C. glabrata* (33.7%), and mild inhibition against *C. krusei* (27.9%) and *C. parapsilosis* (28.2%) and *C. tropicalis*

(27.0%). Upon evaluating the inhibition percentages of Lrh7, relatively moderate values were observed (Table 6). In detail, inhibition value against *C. albicans* was 19.0%, *C. krusei* 28.8%, *C. glabrata* 36.8% and *C. tropicalis* 37.1%, while no inhibitory effect was observed against *C. parapsilosis*.

4.3 Anti-microbial activity against selected pathogens

The results of the cell-free supernatants (CFSs) and cell suspensions of the 32 bacteria strains that have shown anti-microbial activity against 5 pathogenic bacteria by the well diffusion assay method are shown in Table 7. It needs to be noted that the cell-free supernatants (CFS) of all 32 strains did not present any antimicrobial activity against any of the 5 pathogens tested.

Lbr1 strain didn't show any antimicrobial activity except against *Salmonella* with inhibition growth zone of 1.1 cm. Same results are observed in Lbr2, Lbr5 and Lbr6 strains with inhibition growth zone against *Salmonella* being at 1.2, 1.0 and 1.3 cm. Lbr3 and Lbr8 strains exhibited anti-microbial activity only against *E. coli* ATCC 105393 (0.8 cm for both strains) and *Salmonella* with a value of 1.2 cm and 0.3 cm respectively. Strain of Lbr10 revealed antimicrobial activity against *E. coli* ATCC 35218 (0.9 cm) and *Salmonella* (1.2 cm). Lbr11 and Lbr7 strains showed the best results between L. brevis strains, creating inhibition growth zone in three out of five pathogens. In detail, regarding Lbr11, a diameter of 1.2 cm was observed against *E. coli* ATCC 105393, 0.8 cm against *E. coli* ATCC 35218 and 1.4 cm against *Salmonella*. Same with Lbr7, a diameter of 0.6 cm was observed against *E. coli* ATCC 105393 and *E. coli* ATCC 35218 and 1.5 cm against *Salmonella*. No anti-microbial activity was observed in any pathogens for Lbr4 and Lbr9 strain (Table 7).

All *L. delbrueckii* strains exhibited antimicrobial activity against all pathogens except *Listeria monocytogenes*. Notably, Ldb3 revealed relatively better results than the other two strains, with values against *E. coli* ATCC 105393 , *E. coli* ATCC 35218 (Figure 6), *Salmonella* and *S. aureus* DSM 1104 of 1.3 cm, 1.4 cm, 1.8 cm and 1.5 cm respectively. Ldb2 also showed good results against *E. coli* ATCC 105393 (1.9 cm) (Figure 6), *E. coli* ATCC 35218 (1.2 cm), *Salmonella* (1.6 cm) and *S. aureus* DSM 1104 (1.2 cm). Inhibition diameters of Ldb1 were lower but still reasonable. In fact, 0.8 cm

diameter was measured against *E. coli* ATCC 35218 and *S. aureus*, 1.0 cm against *Salmonella* and 0.6 cm against *E. coli* ATCC 105393 (Table 7).

Four out of the eleven *L. plantarum* strains, Lpl1, Lpl2, Lpl3, Lpl4, didn't have any antimicrobial activity against any of the five pathogens. Lpl5 showed a 0.9 cm inhibition zone when tested against *E. coli* ATCC 35218, 1.0 cm against *Salmonella*, and 1.1 cm against *Listeria monocytogenes*. However, no observable inhibition zones were detected against *S. aureus* DSM 1104 or *E. coli* ATCC 105393. Similarly, Lpl6 didn't exhibit any antimicrobial activity against *E. coli* ATCC 105393 or *S. aureus* DSM 1104 while 1.0 cm inhibition zone was observed against *E. coli* ATCC 35218, 1.1 cm against *Salmonella*, and 1.2 cm against *Listeria monocytogenes*.

For the rest of the five *L. plantarum* strains (Lpl7, Lpl8, Lpl9, Lpl10, Lpl11) no inhibition growth zone was observed against *Listeria monocytogenes*. However, all strains showed reasonable results for the remaining pathogens. In detail, Lpl7 exhibited a 1.4 cm inhibition zone when exposed to *E. coli* ATCC 105393, and 1.0 cm when exposed to *S. aureus* DSM 1104. When tested against *Salmonella* and *E. coli* ATCC 35218, a 1.6 cm inhibition zone against both strains was detected. As far as *E. coli* ATCC 105393, inhibition zone of 1.4 cm was observed for Lpl8, Lpl9 and Lpl10 while against *E. coli* ATCC 35218 the diameter was 1.3 cm (Figure 6). However, these strains had different values against *Salmonella* with diameter of 1.5 cm, 1.7 cm and 1.6 cm respectively. Inhibition zone against *S. aureus* DSM 1104 for Lp8 and Lpl10 was 1.0 cm and for Lpl9 0.9 cm. No inhibition growth zone was observed by Lpl11 strain against *S. aureus* DSM 1104 while values against *E. coli* ATCC 35218 (0.8 cm), *E. coli* ATCC 105393 (1.0 cm) and *Salmonella* (1.9 cm) were relatively the same with previous strains.

Table 7. Anti-microbial activity of cell suspensions strains against pathogens evaluated by well diffusion assay. Data indicate inhibition zone diameter (cm).

	***E. coli* ATCC 105393**	***E. coli* ATCC 35218**	***Salmonella***	***Listeria monocytogenes***	***S. aureus* DSM 1104**
Lbr1	-	-	1.1	-	-
Lbr2	-	-	1.2	-	-
Lbr3	0.8	-	1.0	-	-
Lbr4	-	-	-	-	-
Lbr5	-	-	1.0	-	-
Lbr6	-	-	1.3	-	-
Lbr7	0.6	0.6	1.5	-	-
Lbr8	0.8	-	0.3	-	-
Lbr9	-	-	-	-	-
Lbr10	-	0.9	1.2	-	-
Lbr11	1.2	0.8	1.4	-	-
Ldb1	0.6	0.8	1.0	-	0.8
Ldb2	1.9	1.2	1.6	-	1.2
Ldb3	1.3	1.4	1.8	-	1.5
Lpl1	-	-	-	-	-
Lpl2	-	-	-	-	-
Lpl3	-	-	-	-	-
Lpl4	-	-	-	-	-
Lpl5	-	0.9	1.0	1.1	-
Lpl6	-	1.0	1.1	1.2	-
Lpl7	1.4	1.6	1.6	-	1.0
Lpl8	1.4	1.3	1.5	-	1.0
Lpl9	1.4	1.3	1.7	-	0.9
Lpl10	1.4	1.3	1.6	-	1.0
Lpl11	0.8	1.0	1.9	-	-
Lrh1	-	-	-	-	-
Lrh2	1.2	1.3	1.4	-	0.6
Lrh3	1.0	0.8	1.2	-	-
Lrh4	0.8	-	1.2	-	-
Lrh5	-	0.6	-	1.2	-
Lrh6	-	-	-	-	-
Lrh7	-	-	-	1.0	-

As evidenced by Table 7, in two out of seven *L. rhamnosus* strains, Lrh1 and Lrh6, no anti-microbial activity against any of the five pathogens was detected, while Lrh7 exhibited only against *Listeria monocytogenes* a 1.0 cm inhibition zone. Lrh4 and Lrh5 revealed anti-microbial activity against only two pathogens, Lrh4 particularly against *E. coli* ATCC 105393 (0.8 cm) and *Salmonella* (1.2 cm) while Lrh5 against *E. coli* ATCC 35218 (0.6 cm) and *Listeria monocytogenes* (1.2 cm). Lrh3 strain created inhibition zones when exposed to three out of five pathogens, *E. coli* ATCC 105393 (1.0 cm), *E. coli* ATCC 35218 (0.8 cm) and *Salmonella* (1.2 cm). Lrh2 expressed the best results between *L. rhamnosus* strains since inhibiton zones were observed in four out of five pathogens. Notably, diameter of inhibition zones when tested against *E. coli* ATCC 105393, *E. coli* ATCC 35218 and *Salmonella* were 1.2 cm, 1.3 cm and 1.4 cm respectively. Furthermore, Lrh2 was the only strain out of *L. rhamnosus* strains which created inhibition zone (0.6 cm) when exposed to *S. aureus* DSM 1104.

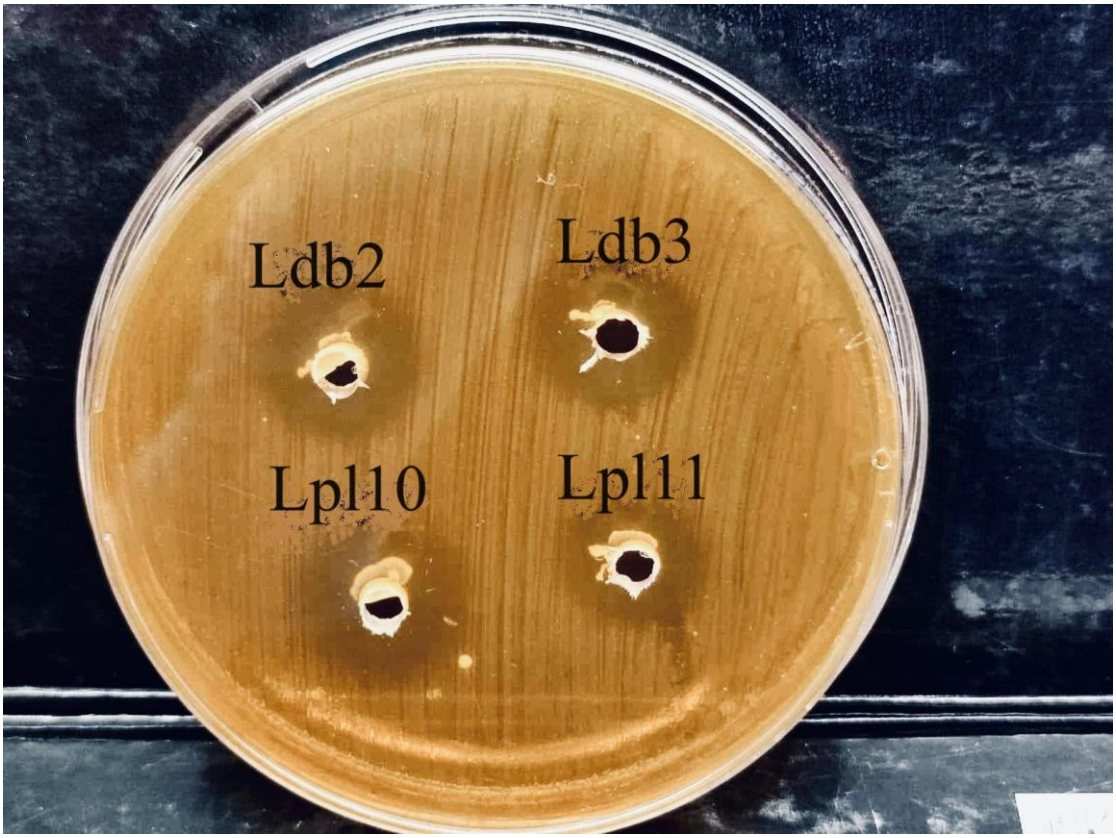

Figure 6. Antimicrobial activity of Ldb2, Ldb3, Lpl10 and Lpl11 strain against *E. coli* ATCC 35218.

4.3 Safety tests

In this study, 3 typical safety parameters were examined to characterize beneficial and probiotic strains intended for potential human and animal consumption. Notably, none of the examined strains exhibited hemolysis, gelatinase or DNase activity in the conducted *in vitro* tests since all tested strains yielded negative results aligning with expectations for safe cultures.

Hierarchical cluster analysis (HCA) was applied to the dataset derived from the different assays to explore the unsupervised discrimination of the diverse probiotic strains into clusters against the selected pathogenic bacteria and probiotic phenotypes (biofilm formation, anti-adhesion activity, and antimicrobial activity). Prior to analysis the data were transformed using Pareto scaling to avoid bias due to differences in scale. HCA was performed based on Euclidean distance as similarity measure and Ward's linkage as clustering algorithm using XLSTAT 2017 (Addinsoft, Paris, France). The results were illustrated in the form of heatmap that is a 2D visualization technique that re-arranges the rows and columns of the data, so that similar rows and similar columns are grouped together and their similarity presented by a dendrogram. Various shades of red and yellow colors in the heatmap correspond to strong and weak similarities between subjects (bacteria strains) and variables (assays), respectively.

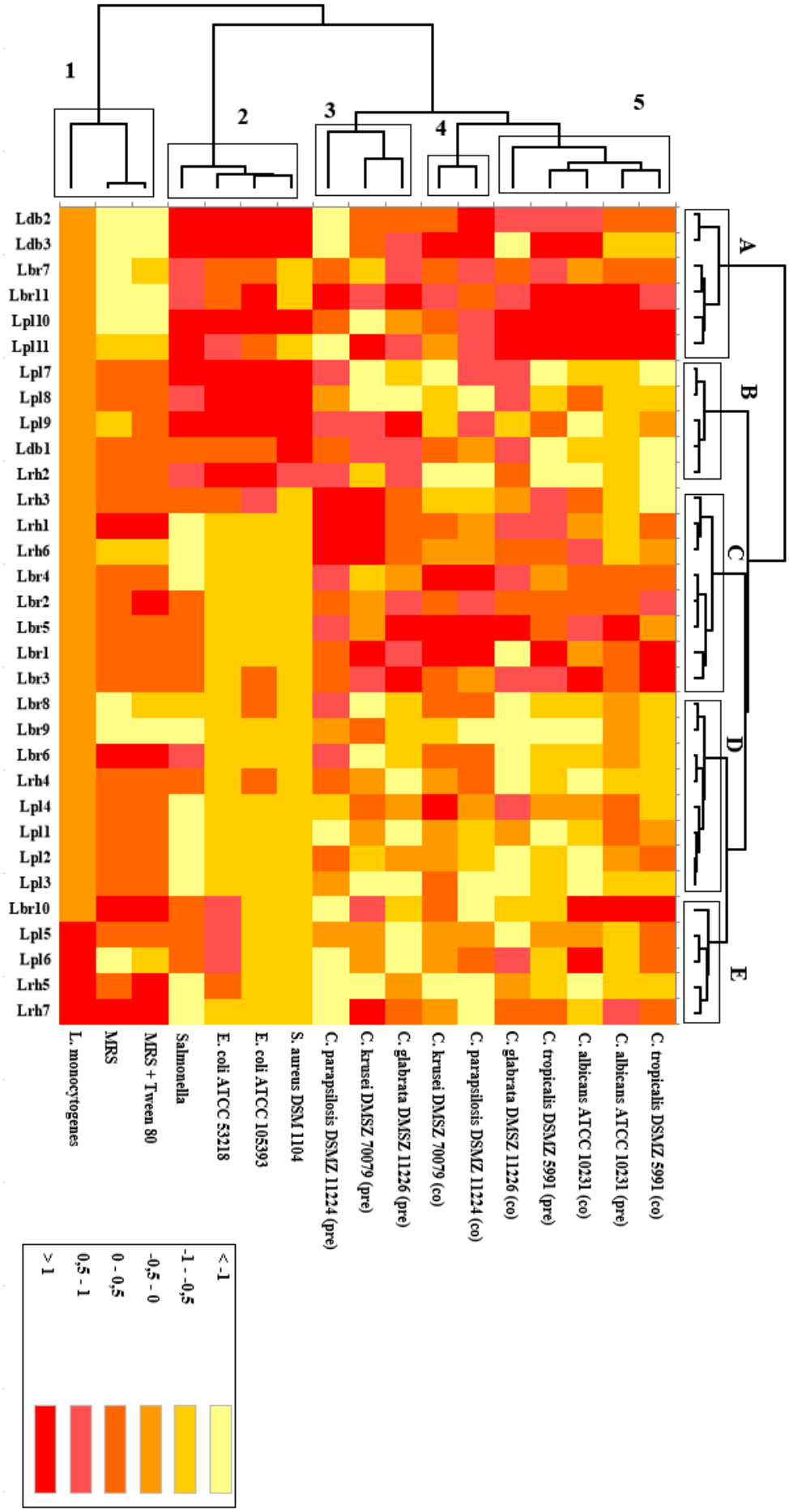

Figure 7. Hierarchical cluster analysis (HCA) of variables (probiotic and technological traits) and subjects (bacteria strains) shown in the form of a heatmap.

The heatmap clustered the 32 bacteria strains into two major clusters and five subclusters (A, B, C, D, and E) as shown in Figure 7. Cluster A included six strains (Ldb2, Ldb3, Lbr7, Lbr11, Lpl10 and Lpl11), cluster B five strains (Lpl7, Lpl8, Lpl9, Ldb1 and Lrh2), cluster C eight strains (Lrh3, Lrh1, Lrh6, Lbr4, Lbr2, Lbr5, Lbr1 and Lbr3), cluster D eight strains (Lbr8, Lbr9, Lbr6, Lrh4, Lpl4, Lpl11, Lpl12 and Lpl13) and cluster E five strains (Lbr10, Lpl5, Lpl6, Lrh5, Lrh7). Probiotic phenotypes were also divided into two major clusters and five subclusters (1, 2, 3, 4, and 5). Cluster 1 included three variables (*L. monocytogenes*, MRS and MRS+Tween80), cluster 2 four variables (*Salmonella*, *E. coli* ATCC 105393, *E. coli* ATCC 35218 and *S. aureus* DSM 1104), cluster 3 three variables (*C. parapsilosis, C. krusei, and C. glabrata* for pre-coating*)*, cluster 4 two variables (*C. krusei,* and *C. parapsilosis* for co-incubation) and cluster 5 five variables (*C. albicans, and C. tropicalis* both for pre-coating and co-incubation as well as *C. glabrata* for co-incubation).

One variable of cluster 1 (*Listeria monocytogenes)* was highly associated with Lpl5, Lpl6, Lrh5 and Lrh7 strains (red colour in the heatmap) belonging to *L. plantarum* and *L. rhamnosus*, indicating that these strains can be effective against *Listeria monocytogenes* compared to the other strains which displayed negative correlation (light orange in the heatmap). The effect of *L. rhamnosus* on the pathogen could be attributed to the production of bacteriocin (rhamnocin). Several studies have underscored the effectiveness of bacteriocins in suppressing *L. monocytogenes*. For instance, the study by Jeong et al. (2015) demonstrated that rhamnocin derived from *L. rhamnosus* notably reduced the cell count of *L. monocytogenes* within a 3-hour period of exposure. Additionally, research conducted by Guerrieri et al. (2009) found that the biofilm created by *L. plantarum* strains could influence the survival and growth of *L. monocytogenes*. Among these strains, *L. plantarum* 35d, known for its production of bacteriocins, showed superior efficacy in diminishing the presence of *L. monocytogenes* when compared to the strains that do not produce bacteriocins. Clusters B, C, D, and E showed positive correlation with Cluster 1. This indicates that Ldb2, Ldb3, Lbr7, Lbr11, Lpl10 and Lpl11 strains could not form strong biofilm by using MRS broth or MRS broth supplemented with 0.1% Tween 80 against *Listeria monocytogenes* compared to the other strains. However, many studies (Shalabi et al., 2022; Tang et al., 2023) have demonstrated the high probiotic properties of *L. delbrueckii*, suggesting its ability to impede the adhesion of pathogenic bacteria. Moreover, Singh et al. (2020)

and Chait et al. (2021) also presented additional proof of the antimicrobial efficacy of *L. brevis* against various pathogenic bacteria.

Two variables of Cluster 1 (MRS and MRS+Tween80) did not present any differentiation between them, indicating that supplementation of Tween 80 did not affect the performance of the bacteria strains. Only in some strains of *L. brevis*, *L. rhamnosus* and *L. plantarum* there was a slight increase of biofilm formation due to the presence of Tween 80. However, the results obtained by Nielsen et al. (2016) highlighted the importance of Tween 80 and the concentration and nature of sugars in the growth medium for biofilm development. Excluding Tween 80 from the MRS medium promoted biofilm formation, whereas a gradual rise in sugar levels led to a notable reduction in biofilm formation.

Clusters A and B were highly associated with Cluster 2 (red and pink colour) showing that the bacteria strains included in this clusters had better antimicrobial activity against *E. coli* ATCC 35218, *E. coli* ATCC 105393, *Salmonella* spp., and *S. aureus* DSM 1104 in comparison with Clusters C, D, and E. Especially bacteria strains from Clusters C, D, and E did not present any antimicrobial activity against *S. aureus* DSM 1104. Most of the strains from Clusters A and B belong to *L. plantarum* indicating that *L. plantarum* can produce biofilm against these pathogens easier. The study of Soleimani et al. (2010) is in agreement with our results, reporting that among the 4 LAB strains investigated, *L. acidophilus*, *L. plantarum*, *L. casei* and *L. reuteri*, *L. plantarum* demonstrated the strongest inhibitory effect against *Staphylococcus aureus*. Liu et al. (2018) also highlighted unique characteristics of *L. plantarum* reaction against *Salmonella* infection. In detail, the findings indicated that various strains of *L. plantarum* exhibited different abilities to inhibit Salmonella growth. This, in turn, prevented pathogens from adhering to and invading epithelial cells, while also boosting immune responses.

Three strains from Cluster C (Lrh3, Lrh1, Lrh6) belonging to *L. rhamnosus* were highly associated with Cluster 3, especially with *C. krusei* DMSZ 70079 and *C. parapsilosis* DSMZ 11224 in the pre-coating assay, revealing that the CFS of Lrh3, Lrh1, Lrh6 were able to hinder the forenamed *Candida* species easier than the rest of the strains. However, Spaggiari et al. (2022) evaluated the CFS derived from *L. acidophilus*, *L. plantarum*, *L. rhamnosus*, and *L. reuteri* for their impact on the virulence characteristics of *C. parapsilosis* and reported that only CFS from *L. rhamnosus* was not able to inhibit *C. parapsilosis* growth. Similarly, in the study of

Jørgensen et al. (2020), *C. krusei* displayed resilience and neutralized the acids generated by the LAB, with *C. krusei* being either unaffected or only slightly inhibited.

Cluster A displayed high association (red and pink colour in the heatmap) with Cluster 5. Specifically, the CFS of Lbr11, Lpl10, and Lpl11 presented high positive correlation with *C. albicans* ATCC 10231 and *C. tropicalis* DSMZ 5991, as well as with *C. glabrata* DMSZ 11226 in co-incubation. This indicates that some strains of *L. brevis* and *L. plantarum* could form biofilm which could hinder better the growth of *C. albicans* ATCC 10231, *C. tropicalis* DSMZ 5991, and *C. glabrata* DMSZ 11226. The obtained results are in agreement with Poon et al. (2023), in which the CFS from *L. plantarum* notably impeded the *in vitro* growth of biofilms produced by *C. albicans* and *C. tropicalis*.

Cluster A exhibited generally the best results for all probiotic phenotypes, displaying the highest association (highly red and pink colours in the heat map) with the clusters corresponding to the various variables (pathogenic bacteria and probiotic assays). In other words, the strains Ldb2, Ldb3, Lbr7, Lbr11, Lpl10 and Lpl11 were the most promising strains to control pathogens and for their potential use in probiotic formulations aimed at combating diverse pathogens in food and feed applications.

5. Conclusions

In recent years, there has been a notable interest regarding the exploration of bacteria species as potential probiotics due to their numerous health benefits. These bacteria are frequently present in fermented foods like yogurt, kefir, and sourdough, and their consumption has been linked to enhanced digestive health, strengthened immune function, and decreased susceptibility to various illnesses. This study focused on *Lacticaseibacillus rhamnosus* isolated from donkey milk, *Lactobacillus delbrueckii* isolated from Pecorino and Ragusano cheese, *Levilactobacillus brevis* isolated from sourdough, and *Lactiplantibacillus plantarum* isolated from sourdough, donkey milk and olives, assessing their capacity to form biofilm against numerous pathogens (*Candida albicans* ATCC 10231, *C. tropicalis* DSMZ 5991, *C. krusei* DMSZ 70079, *C. glabrata* DMSZ 11226, *C. parapsilosis* DSMZ 11224, *E.coli* 105393, *E.coli* 35218, *Listeria monocytogenes*, *Salmonella,* and *S. aureus* DSM 1104).

The heatmap analysis of the 32 bacteria strains revealed distinct clustering patterns, with significant implications for their probiotic and antimicrobial properties. Specifically, the strains Ldb2, Ldb3, Lbr7, Lbr11, Lpl10, and Lpl11 isolated from *L. plantarum*, *L. delbrueckii,* and *L. brevis* demonstrated promising antimicrobial effects against a range of pathogens (*C. tropicalis, C. albicans, C. glabrata, E. coli* ATCC 35218, *E. coli* ATCC 105393, *Salmonella* spp., and *S. aureus* DSM 1104), indicating their potential as effective probiotic candidates. In addition, *L. delbrueckii* and *L. plantarum* can form strong biofilm against *E. coli* ATCC 35218, *E. coli* ATCC 105393, *Salmonella* spp., and *S. aureus* DSM 1104. *L. plantarum* presented also increased inhibition performance against *C. tropicalis,* and *C. albicans*. Finally, *L. plantarum* and *L. rhamnosus,* especially the strains Lpl5, Lpl6, Lrh5, Lrh7 strains, are great probiotic candidates and could inhibit effectively the growth of *Listeria monocytogenes*.

Overall, these findings provide valuable insights into the probiotic and antimicrobial properties of different bacteria strains, with Ldb2, Ldb3, Lbr7, Lbr11, Lpl10, and Lpl11 strains standing out as promising candidates for further investigation and potential utilization in probiotic formulations aiming at combating various pathogens in food and feed applications.

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