Isolation and Characterization of a Probiotic *Bacillus sp.* from Donkey Milk and Its Microencapsulation for Enhanced Gastrointestinal Delivery

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Abstract
Probiotics should have strong attributes to survive across the gastrointestinal tract and be able to colonize. Improving the probiotic stability and targeted delivery remains the major challenge for functional food development. The present paper extracted probiotic bacteria from donkey (*Equus asinus*) milk, identified their functional features, and generated an encapsulation system that presented better stability and loading.Two Gram-positive, catalase-positive bacterial strains were isolated using MRS medium and evaluated for probiotic properties, including auto-aggregation, cell surface hydrophobicity, antimicrobial activity, acid tolerance, and resistance to the simulated gastric fluid. The selected strain was identified as *Bacillus sp*. Microencapsulation was performed using an alginate-xanthan gum matrix by the extrusion method. Encapsulation efficacy, capsule morphology, storage stability, release kinetics in stimulated intestinal fluid, and in vitro toxicity were assessed. *Bacillus sp.*exhibited superior probiotic properties, including auto-aggregation (69.34%), cell surface hydrophobicity (59.4%), DPPH radical scavenging activity (85%), acid tolerance with only a 0.57-log reduction at pH 3, and strong antimicrobial activity against *Serratia sp., Escherichia coli, Staphylococcus aureus, and Klebsiella pneumoniae*. The optimized formulation (10% sodium alginate, 1.5% xanthan gum) achieved 91% encapsulation efficiency and produced spherical capsules of about 3000 mm diameter. Encapsulated cells showed 3.7-log improved survival in simulated gastric fluid at pH 2.0 and 3.03-log higher viability after 50 days of refrigerated storage. PH-responsive release resulted in near complete cell liberation within 4 h in simulated intestinal fluid. In vitro cytotoxicity assay confirmed formulation safety. The developed xanthan gum encapsulation system significantly enhances probiotic survival, storage stability, and targeted intestinal delivery.

Keywords: Probiotic, *Bacillus sp.*, Encapsulation, alginate, xanthan gum, controlled release

Introduction

Gaining significant popularity in recent decades, probiotics are live microorganisms, whose application under sufficient quantity produces several health benefits to the host and thus can be considered as having a potential therapeutic effect (Das et al., 2022). These probiotics have multiple health-promoting functions, including control of intestinal microflora, immunomodulatory, antimicrobial activity against pathogens and relief of most gastrointestinal diseases (Petrariu et al., 2023). The health-relevant benefits of probiotics go beyond intestinal health to include a decrease in cholesterol levels, a decrease in lactose intolerance, reduction of pathogenic organism, as well as a possible prevention of colon cancer (Amin et al., 2025; Latif et al., 2023; Lau and Quek etal., 2024). The selection of probiotic strains should be evaluated against several parameters to deliver safety and effectiveness. The candidate strains should possess the following simple characteristics namely proper Gram reaction, catalase activity profile, and above all, survival under simulated gastrointestinal tract (GIT) conditions (Barzegar et al., 2021). Resistance to the acidity of the stomach (pH 1-3) and exposure to bile salts are critical conditions that define probiotic-activity (Fuochi et al., 2015). In addition, promising strains have to exhibit high concentrations of antagonistic activity against pathogenic bacteria producing organic acids, hydrogen peroxide, and bacteriocins, among other antimicrobial metabolites (Arena et al., 2016). The processes that contribute to probiotic efficacy comprise competitive exclusion of pathogens, blockage of adhesion sites on intestinal mucosa, improvement of mucosal barrier functions, and immunomodulation (Latif et al., 2023b). Natural sources like fermented foods and raw milk are rich sources of isolation of novel probiotic strains with potentially superior properties (Angmo et al., 2016 and Argyri et al., 2013). The most intriguing donkey milk source is known for its nutritional and therapeutic properties, which are reported to have immunological benefits, hypoallergenicity and is traditionally used in treating a number of disorders, such as atherosclerosis and immune-related disorders (Xu

et al., 2025 and Madhusudan et al., 2017). Although nutritional elements have been characterized, the microbial flora of donkey milk has not been well studied, and new strains with distinctive probiotic traits could be discovered. Besides the choice of strains, the optimization of the conditions under which they are cultivated is a crucial consideration in the process of creating commercially viable probiotic products. One of the biggest problems of probiotic use is to preserve cell viability in the storage of the product and in the transition through the hostile gastric environment (Pramanik et al., 2023). Encapsulation technology has been adopted as a viable approach to ensure probiotic cells are spared from environmental pressures (Sun et al., 2023). Biocompatible matrices composed of biopolymers like sodium alginate and xanthan gum are used to protect cells against acidic pH but allow cells to release themselves in an orderly manner in the intestine (Abourehab et al., 2022 and Fazal et al., 2023). The extrusion method allows the creation of spherical capsules with the desirable size range that strikes the necessary balance between protective properties and digestibility (Bennacef et al., 2023 and Lee et al., 2013). This study aimed to isolate and characterize probiotic strains from Equus asinus milk through comprehensive biochemical and physiochemical methodologies, and to develop an encapsulated probiotic formulation with enhanced stability and controlled release properties. Through systematic evaluation of strain characteristics and optimization of encapsulation technology, this research seeks to contribute to the development of novel probiotic products with improved functionality and commercial viability.

Material And Methods

1.1. Isolation and Preliminary Characterization of bacterial Strains

Milk samples of donkey (Equus asinus) were aseptically collected, transported under refrigeration condition and processed immediate upon arrival (Soto Del Rio Mde et al., 2016). The samples were serially diluted using distilled water and spread-plated on De Man, Rogosa and Sharpe (MRS) agar (HiMedia, LM017) (Aneja etal., 2007). The plates were incubated under anaerobic condition at 37°C.Distinct colonies was isolated and purified by repeated streaking and maintained on MRS agar slants 4°C. The Gram staining was done on purified isolates, and the overnight cultures were centrifuged (6,000 rpm, 5 min), washed with sterile water, and heat fixed on glass slides. They were subsequently stained with crystal violet, the iodine of Gram, 95% ethanol (decolorization) and safranin. To check the activity of catalase, a drop of 3% hydrogen peroxide was added to the overnight culture and observed for the formation of bubbles (Hadwan et al., 2024). Species identification was performed based on the morphological and biochemical characteristics. Future research will address the molecular identification, such as the 16sRNA gene sequencing, which was not done in this study

1.2. Aggregation and Hydrophobicity

Auto-aggregation was determined by incubation of washed bacterial cells (suspended in PBS, pH 7.2) at 37°C after 24 h, with measurement taken at 2 h time intervals. Auto-aggregation percentage was determined as: $\left(1 - \left(\frac{OD_{600}\ of\ Upper\ Suspension}{OD_{600}\ of\ Total\ Culture}\right)\right) \times 100$ (Zawistowska-Rojek et al., 2022). Microbial adhesion to hydrocarbons was used to determine cell surface hydrophobicity. Cells (10^8 CFU/mL in 0.1 M KNO_3) were combined with xylene and vortexed (2 min), followed by incubation to separate (20 min). Hydrophobicity was determined: $100 \times \left(\frac{A_0 - A_t}{A_0}\right)$, where A_0 and A_t are the absorbance at 600 nm of the compound prior to and following the treatment with xylene, respectively (Kotzamanidis et al., 2010).

1.3. Antioxidant Activity

The radical scavenging activity of DPPH was assessed through mixing of culture supernatants (100 µL) with ethanol DPPH solution (100 µL), incubating them in darkness (30 min), and recording the absorbance at 517 nm. Similarly, ABTS radical scavenging activity was determined by incubating culture supernatants in darkness with ABTS solution (6-10 min) and recording the absorbance at 734 nm. The scavenging activity was determined as: $\left(\frac{OD_{control} - OD_{sample}}{OD_{control}}\right) \times 100$ (Riaz Rajoka et al., 2017).

1.4. Acid Tolerance and Gastric Survival

In case of acid tolerance, overnight cultures were harvested, then washed twice with PBS (pH 7.2) and resuspended with PBS adjusted to pH 3 or 5. Suspensions were incubated at 37°C, and viable counts were determined after every 1 h by plating on MRS agar (Song et al., 2015). Simulated gastric fluid (SGF; pH 1.99) resistance to NaCl (2g/L), pepsin (3.2g/L), and HCl was also tested. Suspensions of washed cells were incubated at a temperature of 37°C through 3 h, and cells were counted at time intervals (Damodharan et al., 2017, Lee and Salminen, 2009). The results were reported as $\log_{10}$ CFU/mL.

1.5. Antagonistic Activity

Agar well diffusion method was used to evaluate antimicrobial activity against Klebsiella pneumoniae, Proteus mirabilis, Serratia sp, Staphylococcus aureus and Escherichia coli were spread on nutrient agar plate using sterile swabs. Indicator organisms were swab-inoculated to MRS agar plates and wells (6 mm diameter) were punched, after which 100 µL of cell-free culture supernatant was added into each well. After incubating at 37°C overnight for 24 hrs, the positive control was streptomycin measured in millimeters as the inhibition zones (Fijan, 2016).

1.6. Encapsulation

A harvest of *Bacillus sp.* cells in an exponential phase was performed (5,000 rpm, 5 minutes), and the cell suspension was washed twice with PBS (pH 7.2) followed by a 1×10^9 CFU/mL suspension (Shu et al., 2017). Solutions of sterile sodium alginate (10% w/v) and xanthan gum (1.5% w/v) were added together and combined with bacterial suspension (2 mL). A droplet of the mixture was extruded into a 0.1 M CaCl2 solution using a

dropper (0.2 mm needle) and gently stirred. Hardened capsules were filtered and collected in 10 min at room temperature, washed, and stored at 4°C (Vega-Sagardía et al., 2018).

1.7. Physical and Functional Characterization

Stage micrometry was used to measure the diameter of the capsule, and the morphology was observed under optical microscopy. The degree of encapsulation was established by interfering with capsules in PBS, counting the number of released cells through pour plating in MRS agar, and measuring: $\left(\frac{Log_{10}N}{Log_{10}N_0}\right) \times 100$, where N is the released viable cells and N_0 is the initial free cells (Afzaal et al., 2019). The viability of free and encapsulated cells in SGF was measured with incubation of cells in SGF (pH 2.0) at 37°C for 3h, and viable cells were enumerated per hourly following exposure in simulated intestinal fluid (SIF, pH 6.8). Kinetics of release in SIF were analyzed by incubating capsules in SIF at 37°C and counting viable cells every 30 min for 240 min (Pan et al., 2013a). Storage stability was observed by finding viable counts at intervals of 10 days during the 50 days at 10°C of both free and encapsulated cells.

1.8. Safety Assessment

HEp-2 cell monolayers (5 x 10^4 cells/well) were used to assess in vitro cytotoxicity by being exposed to filter-sterilized bacterial supernatants, polymer solutions, and bacteria-polymer mixtures (Rowan et al., 2001; Hong et al., 2008). The incubation was performed overnight (37°, 5% CO_2), after which the WST-1 reagent was added, and absorbance at 450 nm was measured after 4 h incubation. Calculations of cytotoxicity were made as: $\left[1 - \left(\frac{OD_{600}\ of\ Upper\ Suspension}{OD_{600}\ of\ Total\ Culture}\right)\right] \times 100$.

2.9 Ethics statement: Milk sample was collected from the healthy donkeys kept in typical routine farm conditions. The sample process was noninvasive and did not cause any harm to the animal. All procedures adhere to institutional guidelines for animal sample handling.

2.10 Statistical analysis: The findings are shown as the means ± standard deviation, and each experiment was run in triplicate. One-way analysis of variance (ANOVA) and Duncan's multiple range test were used in the statistical study to identify significant differences between the treatments. At $p < 0.05$, the differences were considered statistically significant

Results and Discussion

1.9. Bacterial Isolation from Donkey Milk

Serial dilution and plating of fresh donkey (*Equus asinus*) milk samples on MRS agar resulted in the isolation of several bacterial colonies with different morphological characteristics. Two predominant types of strains were determined based on colony morphology, colony size, colony color and colony texture after 72 h anaerobic incubation at 37°C (Figure 1). The isolates, named Strains 1 and 2, were successfully purified by several repeated quadrant streaking and kept on MRS agar slants for further characterization. Donkey milk has been known to be a promising source for isolation of novel probiotic bacteria, because of its unique composition and associated health benefits (Carroccio et al., 2000 and Živkov Baloš et al., 2023). Previous studies have reported the presence of several lactic acid bacteria in donkey milk, such as *Lactobacillus plantarum*, *Enterococcus faecium*, *Lactobacillus delbrueckii*, *Pediococcus acidilactici*, *Lactobacillus fermentum* and *Bifidobacterium* species (Murua et al., 2013). The isolation of different bacterial strains from this source is in line with the current literature, indicating that donkey milk contains a diverse microbial flora with probiotic properties. The relatively smaller number of different strain types in the current study (two predominant strains) in comparison to some previous reports may be explained by the sampling location, animal diet, lactation stage, or selective nature of the MRS medium used.

1.10. Gram Staining and Morphological Characterization

Microscopic examination after Gram staining showed that both Strain 1 and Strain 2 were Gram-positive organisms in the form of blue-purple rods as detected by light microscopy at 1000x magnification. The Gram-positive nature reflected the presence in the cell wall of a thick layer of peptidoglycan which retained the crystal violet and iodine complex during the decolorization step (Zheng et al., 2020). The rod-shaped morphology was suggestive of the isolates being of the genera *Lactobacillus and Bacillus* (both of which are associated with probiotic application). Gram staining is a basic first step in the characterization of bacteria and gives quick preliminary taxonomic information. The Gram-positive reaction is a prerequisite for most probiotics' bacteria, as most of the established probiotic bacteria belong to Gram-positive genera consisting of *Lactobacillus, Bifid bacterium, Bacillus, and Enterococcus* (Salvetti et al., 2012). Both rod-shape morphology in both isolates is consistent with bacilli, and they are abundant in fermented dairy products and have features amenable to probiotic applications (Angmo et al., 2016).

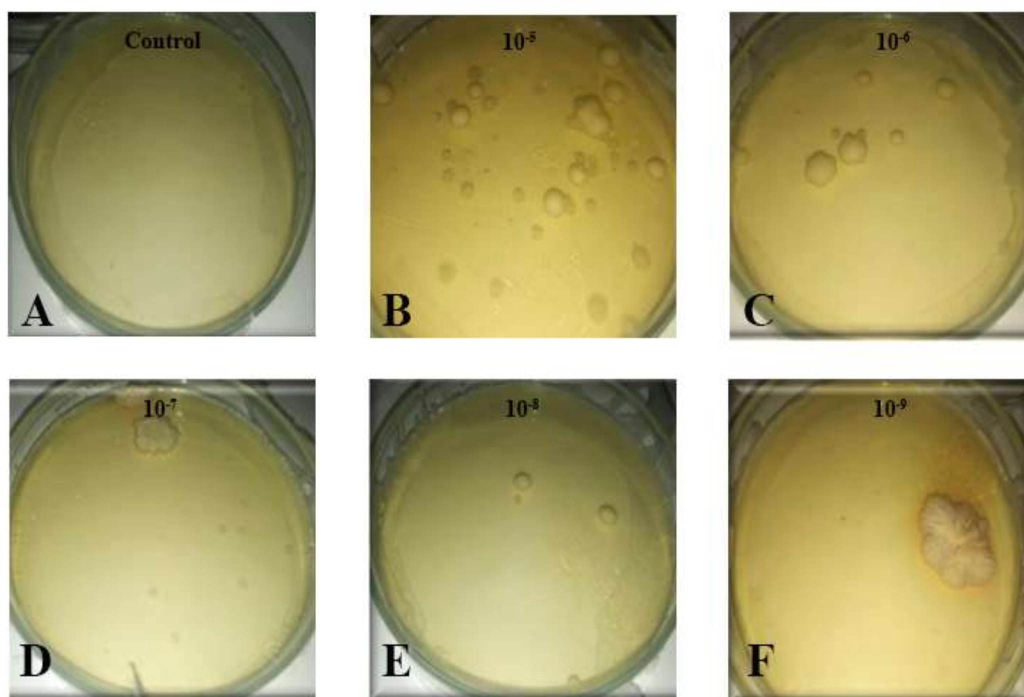

Figure 1 Colonies of strains from *Equus asinus* milk dilution

1.11. Catalase Activity

Both isolates had positive catalase activity as shown by the formation of instantaneous and vigorous bubbles when 3% hydrogen peroxide was added to bacterial cultures (Figure 2). The catalase **-** positive reaction indicated that both isolates contained the enzyme catalase that catalyzes the decomposition of hydrogen peroxide into water and molecular oxygen according to the reaction: $2H_2O_2$ - $2H_2O + O_2$ (Murua et al., 2013).

This catalase-positive is of especially significance in the taxonomic differentiation of the Gram-positive bacteria. Most *Lactobacillus species* are catalase-negative because they lack the catalase enzyme, which contains heme and usually utilizes other mechanisms to handle oxidative stress (Sana et al., 2023). The positive catalase reaction in both isolates therefore strongly suggested that these strains did not belong to the genus *Lactobacillus* despite the initial morphological similarities. There after combination of Gram-positive rods with catalase activity is characteristic of the *Bacillus genus* members which are facultative anaerobes or obligate aerobes that are able to perform aerobic respiration (Sorokulova et al., 2008).

Figure 2 Catalase activity of Strain 1 and Strain 2 showing differential bubble formation

The presence of catalase in bacterial cells has an important protective purpose in neutralizing hydrogen peroxide, a harmful reactive oxygen species that can cause damage to cell components such as DNA, proteins and lipids (Nandi et al., 2019). For probiotic applications there may be an added benefit to the catalase activity, as it may protect the bacterial cell from oxidative stress during processing and storage and during passage through the gastrointestinal tract. *Bacillus species* have attracted growing interest as candidates for probiotic use because of their ability to form spores, which can confer outstanding resistance to adverse environmental conditions such as heat, acidity and desiccation (Mingmongkolchai and Panbangred, 2018). The spore form enables Bacillus probiotics to better tolerate gastric acidity than vegetative cells of lactic acid bacteria and may facilitate their passage for delivery to the intestinal tract where they can germinate and have beneficial effects (Casula and Cutting, 2002).

The preliminary characterization results including Gram-positive rod morphology and positive catalase activity gave strong preliminary evidence that the isolated strains were genera of the *Bacillus species* instead of Lactobacillus or other catalase-negative genera. This finding is especially noteworthy considering the rarity with

which *Bacillus species* are reported from donkey milk compared to the occurrence of lactic acid bacteria, intimating that the isolates could represent novel or underexplored strains with potential probiotic properties. *Bacillus species* have been successfully used as probiotics in different applications and have shown benefits such as the modulation of the immune system, antimicrobial activity against pathogens, the improvement of the digestive enzyme activity and the improvement of the growth performance in human and animal models (Duc et al., 2004, Hong et al., 2008, Liu et al., 2017).

1.12. Auto-aggregation and Cell Surface Hydrophobicity

Auto-aggregation and cell surface hydrophobicity are commonly accepted to be key factors of probiotic adhesion, which determine the capacity of bacteria to colonize the gastrointestinal tract and form long-term persistence (Del Re et al., 2000). In the current investigation, the two traits were quite different in the two isolates (Figure 3). Strain 1 showed a relatively low auto-aggregation capacity of just 16.77% with a 24 h period, whereas Strain 2 showed a significantly higher level of aggregation of 69.34% in the same period.

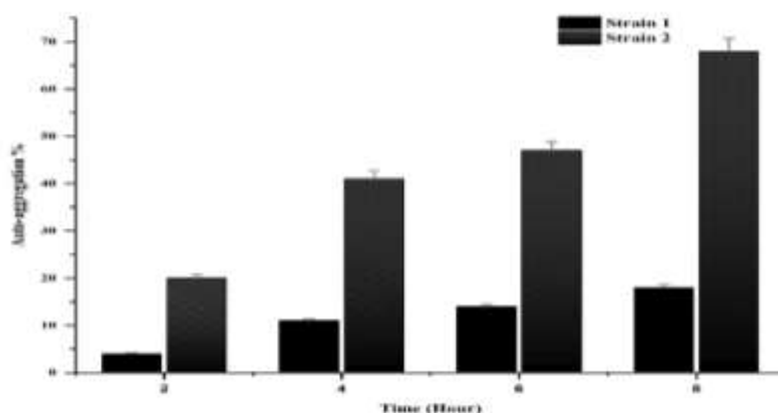

Figure 3 Percentage auto-aggregation of Strain 1 and Strain 2 recorded at a 2-hour interval

The gradual increase in aggregation of Strain 2 indicates that the Strain exhibits strong cell-cell interactions, a characteristic known to enhance mucosal adhesion, biofilm formation and competitive exclusion of pathogenic microorganisms (Collado et al., 2008). The xylene hydrophobicity test further discriminated against the strains. Strain 1 was moderately hydrophobic (35.1%), whereas Strain 2 was 59.4% and well above the 40% mark generally considered to be a target level of probiotic adhesion (Kotzamanidis et al., 2010). Hydrophobicity is associated with the capacity of bacterial cells to associate with the host epithelial membranes and mucosal surfaces; hence, the high hydrophobicity of Strain 2 strengthens its intestinal colonization potential. Taken together, these results indicate that Strain 2 has a better adhesion phenotype than Strain 1, which is not only necessary to colonize but also to promote long-term probiotic effects such as immune regulation, pathogen rejection, and barrier-enhancing effects (Latif et al., 2023a).

1.13. Antioxidant Activity

The DPPH and ABTS radical scavenging assay was used to determine the antioxidant activity that differed significantly among the isolates (Figure 4). Strain 1 had a moderate radical scavenging activity (45%), and Strain 2 had a strong activity (85%) for DPPH. Similarly, strain 1 exhibited 32%, and strain 2 showed 76% against ABTS. The antioxidant effects of probiotics have also continued to raise interest because of the role of oxidative stress in gastrointestinal diseases like inflammatory bowel disease, mucosal inflammation, and cell ageing (Riaz Rajoka et al., 2017). Bacteria that can neutralise reactive oxygen species can thus provide other protective roles other than the normal probiotic processes. The antioxidant activity of Strain 2 could be explained by the formation of antioxidant metabolites or metal-chelating molecules or by enzyme-defenses (such as superoxide dismutase and glutathione peroxidase) (Siragusa et al., 2007).

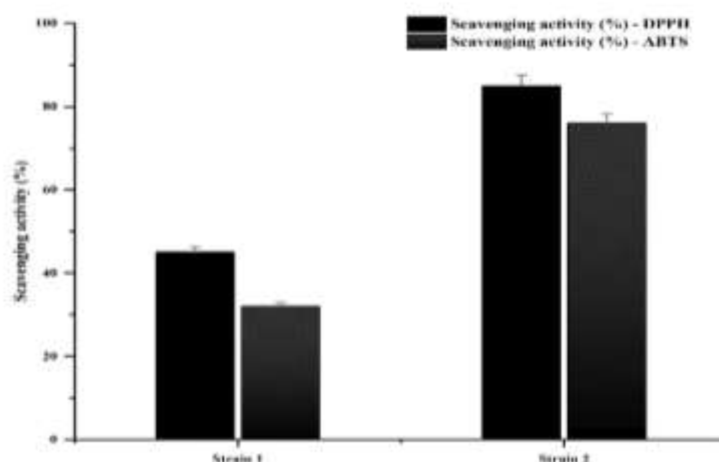

Figure 4 DPPH and ABTS scavenging activities of Strain 1 and Strain 2

These biochemical characteristics could increase the therapeutic utility of the strain by decreasing the level of oxidative stress in the intestinal environment and promoting the stability of the epithelial barrier. On comparison, the intermediate activity of Strain 1 indicates an even more restricted role in this aspect.

1.14. Acid Tolerance

Acid tolerance is crucial to the survival of orally administered probiotics through the stomach tract. Figure 5 shows at pH 5, Strain 1 had a gradual decrease in viability for the 3 h exposure time, with a cumulative loss of 1.6 log units. However, it did not survive at pH 3 which indicates the susceptibility to strongly acidic environments. Conversely, Strain 2 put up an exceptional tolerance to pH alteration of both pH 5 and pH 3, losing only 0.57 log units of pH at pH 3 while maintaining high viable cell numbers (>10^{10} CFU/mL). It is of particular significance that these findings are made since the pH may drop to as low as 1.5 in the stomach during digestion and that bacterial survival must be maintained for successful colonization in the intestine (Prasad et al., 1998). The superior acid tolerance of Strain 2 is consistent with the inherent physiological robustness of Bacillus species, in particular the ability to produce dormant spores resistant to extreme environmental conditions such as low pH, heat and desiccation (Casula and Cutting etal., 2002).

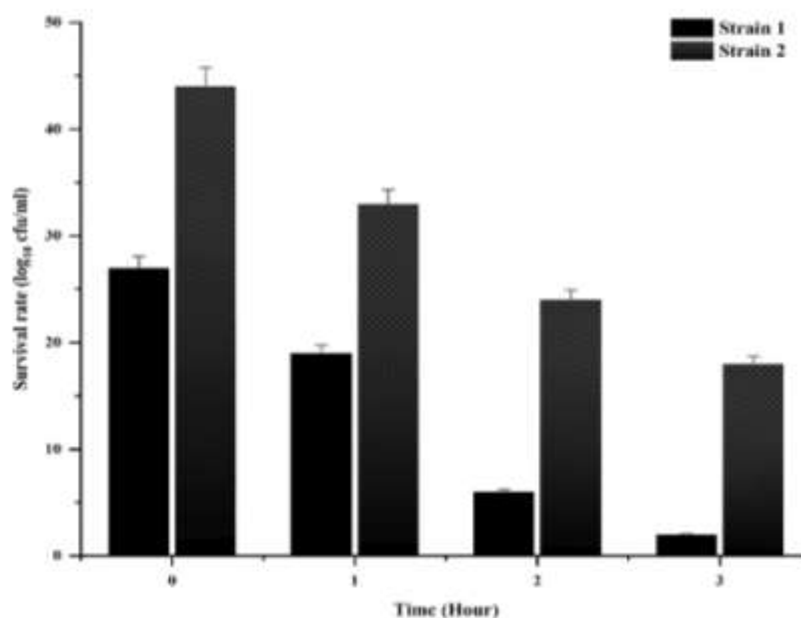

Figure 5 Survival rate of Strain 1 and Strain 2 at low pH expressed as $\log_{10}$ CFU/ml

Spores passing through the stomach can in turn germinate in the small intestine to produce metabolically active vegetative cells which are capable of colonization. This gives a significant edge over many non-spore-forming probiotic species which often have low acid stability. The further acid tolerance results show Strain 2 to be a strong candidate for oral probiotic formulations.

1.15. Antagonistic Activity Against Pathogens

Figure 6 Inhibition zones are shown for: (A-B) *Serratia* sp.; (C-D) *Escherichia coli*; (E-F) *Staphylococcus aureus*; (G-H) *Klebsiella pneumoniae*; and (I-J) *Proteus mirabilis*

Antagonistic activity against gastrointestinal pathogens is a fundamental probiotic function (Figure 6). Strain 1 showed a low level of activity and inhibited only two pathogens: E. coli (13 ±1 mm) and P. mirabilis (20 ±1 mm). In contrast, Strain 2 showed a wide range of antimicrobial activity against four out of five pathogenic strains tested which were *Serratia sp, E. coli, S. aureus and K. pneumoniae* with inhibition zones values of 30 ± 2 mm, 14 ± 1 mm, 25 ± 1 mm, and 30 ± 2 mm respectively (Table1). The wide-ranging activity may indicate that Strain 2 secretes a wide range of antimicrobial molecules including lipopeptides, bacteriocins, and organic acids, which have been well-documented in Bacillus sp. (Urdaci et al., 2004 and Fijan etal., 2016). The antagonistic profile of Strain 2 suggests high potential for pathogen suppression, competitive exclusion and microbiota stabilization. Although Strain 1 showed significant inhibition on P. mirabilis, the wider action of Strain 2 makes it the better candidate for probiotic use for the control of opportunistic pathogens involved in gut dysbiosis.

Table 1 Antagonistic activity of bacillus isolates against pathogenic microorganism

Indicator microorganism	**Diameter of inhibition zones (mm)**		
	Control	Strain 1	Strain 2
Serratia Sp.	33±3	-	31±2
Escherichia coli	32±1	14±1	12±1
Staphylococcus aureus	37±2	-	26±1
Klebsiella pneumoniae	36±2	-	31±2
Proteus mirabilis	19±1	21±1	-

1.16. Resistance to Simulated Gastric Fluid

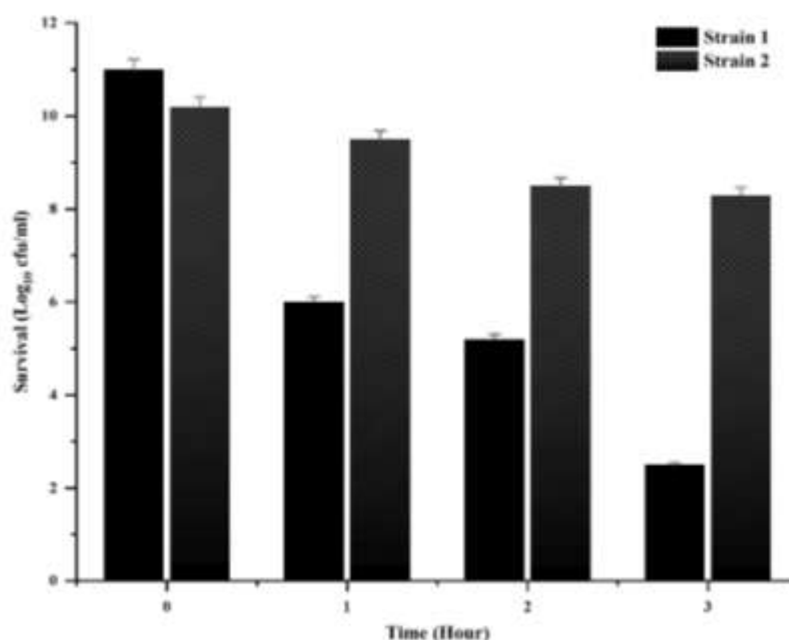

Figure 7 Survival of Strain 1 and Strain 2 in simulated gastric fluid expressed as $\log_{10}$ CFU/ml over time

Tests of SGF (Figure 7) with the addition of both acidic conditions (pH 1.99) and pepsin give the strict test of survival in gastric relevant conditions (Damodharan et al., 2017). Strain 1 exhibited significant changes in viability, which was in line with its low survival in pH 3. Strain 2 had a high viability during 3 h of SGF exposure proving exceptional gastric toughness. Resistance to both low pH and the action of proteolytic enzymes is essential in ensuring that the intestine is colonized by a high percentage of living probiotic cells (Lee and Salminen, 2009). In all assays involving adhesion characteristics, antioxidant capabilities, acid and SGF tolerance, and pathogen inhibition Strain 2 had a superior performance over Strain 1. Its good physiological health, high antimicrobial potential and good adhesion properties all make Strain 2 an attractive probiotic candidate that will require further research such as molecular characterization, in vivo confirmation, and formulation.

1.17. Encapsulation Optimization and Capsule Characterization

Several combinations of polymers were tested to determine conditions that would produce stable capsules with high cell retention. Lower concentrations of polymer (0.5-1% xanthan gum and 2-4% sodium alginate) gave weak capsules with poor structural integrity, whereas higher concentrations (2% xanthan and 8% alginate) gave rigid gels (Figure 8). The optimised formulation 1.5% xanthan gum with 10% alginate produced mechanically stable, spherical capsules suitable for gastrointestinal delivery.

Figure 8: Eencapsulation of *Bacillus sp.* cells with polymeric matrices

The capsule diameter was approximately ~3000 µm, which aligns with the suggested size in extrusion-based encapsulation (100–10,000 µm), whereby capsule size was the balance between protection and controlled degradation (Krasaekoopt et al., 2003; Afzaal et al., 2019). Microscopic assessment revealed encapsulation of the matrices in a microscale form, with the cellular material rod-shaped and evenly distributed throughout the polymer matrix (Figures 9 and 10), a morphology known to facilitate uniform and sustained release (Jafari et al., 2008). The encapsulation efficiency obtained in the study 91% is comparable to or higher than values reported in the previous alginate-based probiotic encapsulation systems, 70 – 90%, depending on the polymer composition and the extrusion parameters (Sultana et al., 2000), and the cell entrapment was excellent and loss during gelation and washing was minimal. This high efficiency is an indication of efficient Ca2+-mediated ionic crosslinking of alginate to produce a dense hydrogel network that can entrap bacterial cells (Sultana et al., 2000, Vega-Sagardía et al., 2018).

Figure 9: Diameter of the capsule measured using a ruler

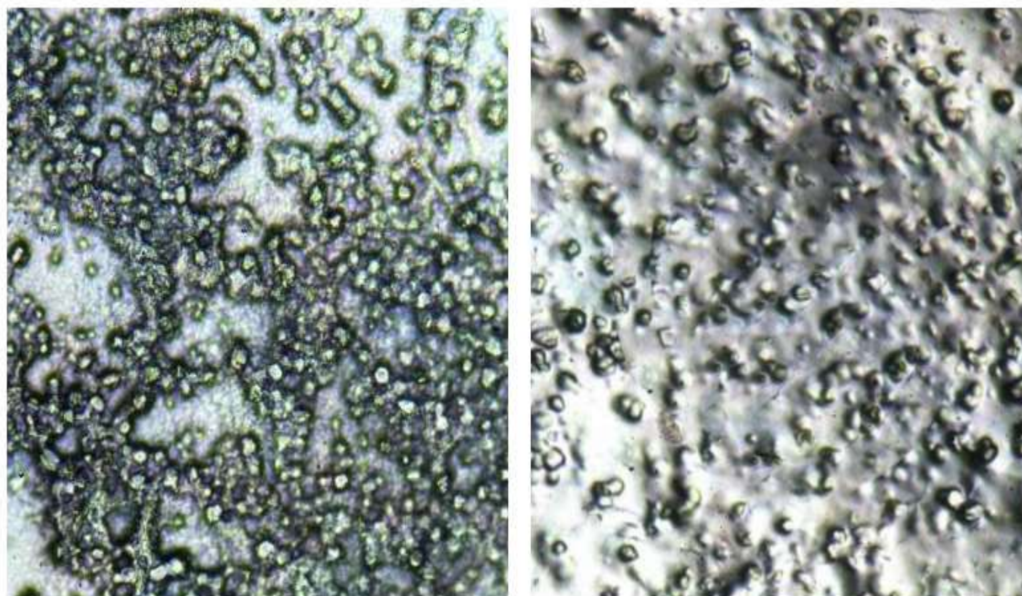

Figure 10 Microscopic images of probiotics encapsulated with sodium alginate and xanthan gum

1.18. Protection in Simulated Gastric Conditions

The protective capacity of encapsulation was tested using free and encapsulated Bacillus sp. cells in SGF (pH 2.0) after 3 hours (Figure 4.11). Free cells showed high and quick viability loss, which began at the initial 11 $\log_{10}$ CFU/mL at 0 h to the final 5 $\log_{10}$ CFU/mL at 3 h, a 6-log loss. Conversely, encapsulated cells demonstrated much greater cell viability, with a result of 8-log10 CFU/mL decrease in CFU/mL up to 10.3 $\log_{10}$ CFU/mL in 60 minutes, which is only 2.3-log. This difference in the survival of encapsulated and free cells of 3.7 logs just goes to indicate that the alginate-xanthan gum matrix provides a significant level of protection against gastric acidity and pepsin activity. The physical barrier formed by the polymer matrix inhibits diffusion of hydrogen ions and pepsin into the inner part of the capsule and ensures a less hostile microenvironment of bacterial cells (Chávarri et al., 2010 and Dong et al., 2013). Even though Bacillus spores do have intrinsic acid resistance, encapsulation gives further protection to the spores and any vegetative cells that may be present, making them have higher survival rates overall through gastric transit. The dense hydrogel network formed by calcium crosslinking of alginate with xanthan gum restricts hydrogen ions and digestive enzymes from entering the capsule, enhancing encapsulated cell survival.

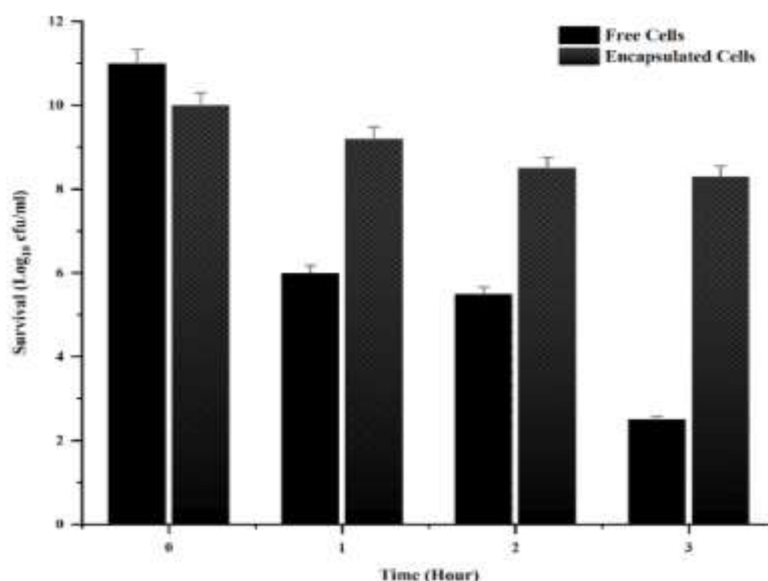

Figure 11 Survival of free and encapsulated *Bacillus sp.* cells in simulated gastric fluid (pH 2.0) over 3 hours

1.19. Release Kinetics in Simulated Intestinal Fluid

Bacillus sp. Biphasic kinetics were shown by the release of encapsulated *Bacillus sp.* in SIF (pH 6.8) at 37°C (Figure 12). The first phase of rapid release was during the first 60 min, which was then followed by a gradual further release over the next 180 min. At 240 min (4 h), there seemed to be a release of about 10 $\log_{10}$ CFU/mL cells, and the initial concentration (10.3 $\log_{10}$ CFU/mL) was almost reached; the cell has almost been totally released. This probiotic has a physiologically suitable profile of release. The presence of pH-responsive behaviours of the alginate-xanthan gum matrix allows keeping the cells trapped under the acidic gastric conditions (pH 2) and releasing them under the neutral-to-slightly-alkaline conditions of the intestines (pH 6-7).

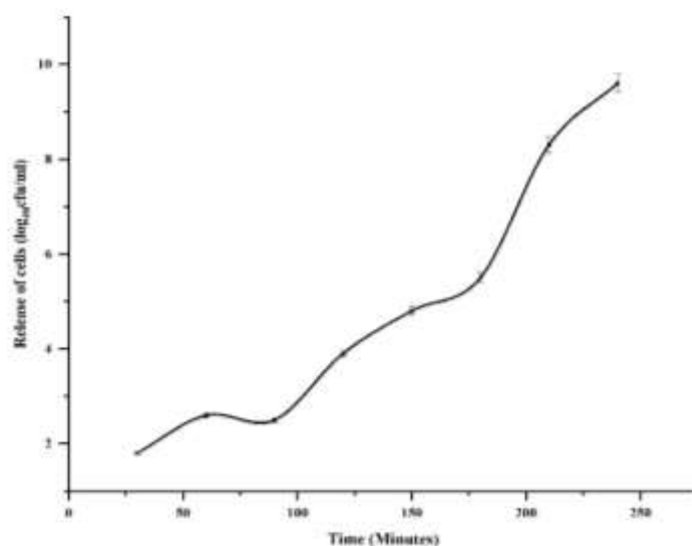

Figure 12 Release profile of encapsulated *Bacillus sp.* in pH 6.8 SIF

The release mechanism is characterized by a gradual dissolution and swelling of the polymer matrix due to an increase in pH, and possible degradation of the polymer by intestinal enzymes (Pan et al., 2013b). The biphasic release pattern-quick initial release accompanied by gradual release- could be beneficial in that it not only offers instantaneous colonization of the proximal areas of the intestine but also offers continuous release of the intestinal areas as the capsules pass through the gastrointestinal tract. Cells that may be present, making them have higher survival rates overall through gastric transit.

1.20. Safety Assessment

Evaluation of in vitro cytotoxicity by using HEp-2 cells monolayers showed that *Bacillus sp.* cells, polymer solutions (alginate-xanthan gum) and bacteria-polymer blends showed no significant cytotoxicity at the concentrations (5-50 µg /ml) tested (Figure 4.23). The viability of the cells in all test conditions was >95% compared to negative controls, which was used to confirm that there was no toxic effect. This safety profile aligns with what has been previously reported, that Bacillus sp. is Generally Recognized as Safe (GRAS) to be consumed by humans, and that alginate-based polymers are biocompatible, non-toxic encapsulation material types that are approved to be utilised in food products (Sorokulova et al., 2008;Hong et al., 2008)

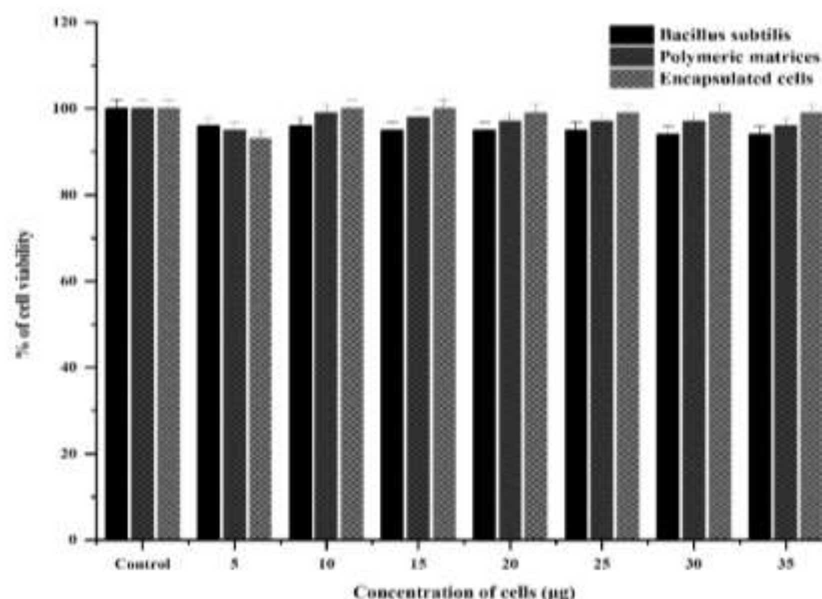

Figure 13 In vitro toxicity assay of *Bacillus sp.* and polymeric matrices against the normal HEp-2 cell line

Conclusion

This study has been able to isolate Bacillus sp. in donkey (Equus asinus) milk with potential probiotic properties, with auto-aggregation (69.34%), hydrophobicity (59.4%), antioxidant properties (85%), acid tolerance (0.57-log reduction at pH 3 during 3 h), and antimicrobial properties against four pathogenic bacteria. The isolate showed much greater probiotic potential than did a second strain when all parameters were considered. A functional encapsulation system of alginate-xanthan gum (10% sodium alginate, 1.5% xanthan gum) was obtained with an encapsulation efficiency of 91% and showed great practical advantages: 3.7-log longer gastric retention than free cells, 3.03-log higher storage stability for 50 days, and pH-controlled release in intestinal conditions. The non-

toxicity of all the formulation components was ascertained through safety assessment. The dual protection of bacteria is an achievement of the synergistic combination of Bacillus spore-forming capacity and polymer encapsulation, which is more effective than traditional probiotics. This study confirms donkey milk as a promising source of new probiotic strains and shows that capsulation with the help of alginate-xanthan gum will help to increase the stability of probiotics, their shelf-life, and targeted delivery to the intestine. The formulation obtained has a high level of commercial feasibility in terms of functional food applications. The next step in the evolution of this probiotic formulation should be future work on the validation of molecular identification and invivo efficacy evaluation, immunomodulatory characterization, and optimisation of large-scale production to realize the future of this probiotic formulation in commercial applications. Encapsulation techniques help incorporate probiotic Bacillus strains into functional dairy products, drinks, or nutraceuticals. These techniques are important when long-term stability and targeted intestinal delivery are needed for therapeutic efficacy.

Statements and Declarations
The authors declare that there is no conflict of interest regarding the publication of this manuscript.

Data Availability Statement
All data analysed during this study are included in this manuscript and the associated supporting information files.

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References

1. Abourehab, M.A.S., Rajendran, R.R., Singh, A., Pramanik, S., Shrivastav, P., Ansari, M.J., Manne, R., Amaral, L.S., and Deepak, A. 2022. Alginate as a promising biopolymer in drug delivery and wound healing: A review of the state-of-the-art. International Journal of Molecular Sciences, 23. https://doi.org/10.3390/ijms23052785
2. Afzaal, M., Saeed, F., Arshad, M.U., Nadeem, M.T., Saeed, M., and Tufail, T. 2019. The effect of encapsulation on the stability of probiotic bacteria in ice cream and simulated gastrointestinal conditions. Probiotics and Antimicrobial Proteins, 11, 1348–1354. https://doi.org/10.1007/s12602-018-9475-8
3. Amin, M.R., Biswas, A.P., Tasnim, M., Islam, M.N., and Azam, M.S. 2025. Probiotics and their applications in functional foods: A health perspective. Applied Food Research, 5, 101193. https://doi.org/10.1016/j.afres.2025.101193
4. Aneja, K. 2007. Experiments in Microbiology, Plant Pathology and Biotechnology. New Age International Publishers.
5. Arena, M.P., Silvain, A., Normanno, G., Grieco, F., Drider, D., Spano, G., and Fiocco, D. 2016. Use of Lactobacillus plantarum strains as a bio-control strategy against food-borne pathogenic microorganisms. Frontiers in Microbiology, 7, 464. https://doi.org/10.3389/fmicb.2016.00464
6. Barzegar, H., Alizadeh Behbahani, B., and Falah, F. 2021. Safety, probiotic properties, antimicrobial activity, and technological performance of Lactobacillus strains isolated from Iranian raw milk cheeses. Food Science & Nutrition, 9, 4094–4107. https://doi.org/10.1002/fsn3.2365
7. Bennacef, C., Desobry-Banon, S., Probst, L., and Desobry, S. 2023. Alginate core-shell capsules production through coextrusion methods: Principles and technologies. Marine Drugs, 21, 235. https://doi.org/10.3390/md21040235
8. Carroccio, A., Cavataio, F., Montalto, G., D'Amico, D., Alabrese, L., and Iacono, G. 2000. Intolerance to hydrolysed cow's milk proteins in infants: Clinical characteristics and dietary treatment. Clinical and Experimental Allergy, 30, 1598–1603. https://doi.org/10.1046/j.1365-2222.2000.00949.x
9. Casula, G., and Cutting, S.M. 2002. Bacillus probiotics: Spore germination in the gastrointestinal tract. Applied and Environmental Microbiology, 68, 2344–2352. https://doi.org/10.1128/AEM.68.5.2344-2352.2002
10. Chávarri, M., Marañón, I., Ares, R., Ibáñez, F.C., Marzo, F., and Villarán, M.D.C. 2010. Microencapsulation of a probiotic and prebiotic in alginate–chitosan capsules improves survival in simulated gastrointestinal conditions. International Journal of Food Microbiology, 142, 185–189. https://doi.org/10.1016/j.ijfoodmicro.2010.06.022
11. Collado, M.C., Meriluoto, J., and Salminen, S. 2008. Adhesion and aggregation properties of probiotic and pathogen strains. European Food Research and Technology, 226, 1065–1073. https://doi.org/10.1007/s00217-007-0632-x
12. Damodharan, K., Palaniyandi, S.A., Yang, S.H., and Suh, J.W. 2017. Co-encapsulation of lactic acid bacteria and prebiotic with alginate–fenugreek gum–locust bean gum matrix. Biotechnology and Bioprocess Engineering, 22, 265–271. https://doi.org/10.1007/s12257-016-0640-3
13. Das, T.K., Pradhan, S., Chakrabarti, S., Mondal, K.C., and Ghosh, K. 2022. Current status of probiotics and related health benefits. Applied Food Research, 2, 100185. https://doi.org/10.1016/j.afres.2022.100185
14. Del Re, B., Sgorbati, B., Miglioli, M., and Palenzona, D. 2000. Adhesion, autoaggregation and hydrophobicity of 13 strains of Bifidobacterium longum. Letters in Applied Microbiology, 31, 438–442. https://doi.org/10.1046/j.1472-765x.2000.00845.x
15. Dong, Q.Y., Chen, M.Y., Xin, Y., Qin, X.Y., Cheng, Z., Shi, L.E. y Tang, Z.X. 2013. Alginate-based and protein-based materials for probiotics encapsulation: A review. International Journal of Food Science & Technology, 48, 1339–1351. https://doi.org/10.1111/ijfs.12097

16. Duc, L.H., Hong, H.A., Barbosa, T.M., Henriques, A.O., and Cutting, S.M. 2004. Characterisation of Bacillus probiotics available for human use. Applied and Environmental Microbiology, 70, 2161–2171. https://doi.org/10.1128/AEM.70.4.2161-2171.2004
17. Fazal, T., Murtaza, B.N., Shah, M., Iqbal, S., Rehman, M.U., Jaber, F., Dera, A.A., Awwad, N.S., and Ibrahim, H.A. 2023. Recent developments in natural biopolymer-based drug delivery systems. RSC Advances, 13, 23087–23121. https://doi.org/10.1039/D3RA04023K
18. Fijan, S. 2016. Antimicrobial effect of probiotics against common pathogens. In: Probiotics and Prebiotics in Human Nutrition and Health, 577–592. https://doi.org/10.5772/63174
19. Fuochi, V., Petronio, G.P., Lissandrello, E. and Furneri, P.M. 2015. Evaluation of resistance to low pH and bile salts of human Lactobacillus spp. isolates. International Journal of Immunopathology and Pharmacology, 28, 426–433. https://doi.org/10.1177/0394632015585993
20. Hadwan, M.H., Hussein, M.J., Mohammed, R.M., Hadwan, A.M., Saad Al-Kawaz, H., Al-Obaidi, S.S., and Al-Talebi, Z.A. 2024. An improved method for measuring catalase activity in biological samples. Biology Methods and Protocols, 9, bpae015. https://doi.org/10.1093/biomethods/bpae015
21. Hong, H.A., Huang, J.M., Khaneja, R., Hiep, L., Urdaci, M., and Cutting, S. 2008. The safety of Bacillus subtilis and Bacillus indicus as food probiotics. Journal of Applied Microbiology, 105, 510–520. https://doi.org/10.1111/j.1365-2672.2008.03773.x
22. Jafari, S.M., Assadpoor, E., He, Y., and Bhandari, B. 2008. Encapsulation efficiency of food flavours and oils during spray drying. Drying Technology, 26, 816–835. https://doi.org/10.1080/07373930802135972
23. Kotzamanidis, C., Kourelis, A., Litopoulou-Tzanetaki, E., Tzanetakis, N., and Yiangou, M. 2010. Evaluation of adhesion capacity of probiotic Lactobacillus strains. International Journal of Food Microbiology, 140, 154–163. https://doi.org/10.1016/j.ijfoodmicro.2010.03.016
24. Krasaekoopt, W., Bhandari, B., and Deeth, H. 2003. Evaluation of encapsulation techniques of probiotics for yoghurt. International Dairy Journal, 13, 3–13. https://doi.org/10.1016/S0958-6946(02)00155-3
25. Latif, A., Shehzad, A., Niazi, S., Zahid, A., Ashraf, W., Iqbal, M.W., Rehman, A., Riaz, T., Aadil, R.M., Khan, I.M., Özogul, F., Rocha, J.M., Esatbeyoglu, T., and Korma, S.A. 2023a. Probiotics: Mechanism of action, health benefits and their application in food industries. Frontiers in Microbiology, 14. https://doi.org/10.3389/fmicb.2023.1216674
26. Latif, A., Shehzad, A., Niazi, S., Zahid, A., Ashraf, W., Iqbal, M.W., Rehman, A., Riaz, T., Aadil, R.M., Khan, I.M., Özogul, F., Rocha, J.M., Esatbeyoglu, T., and Korma, S.A. 2023b. Probiotics: Mechanism of action, health benefits and their application in food industries. Frontiers in Microbiology, 14, 1216674. https://doi.org/10.3389/fmicb.2023.1216674
27. Lau, L.Y.J., and Quek, S.Y. 2024. Probiotics: Health benefits, food application, and colonisation in the human gastrointestinal tract. Food Bioengineering, 3, 41–64. https://doi.org/10.1016/j.fbioe.2024.100041
28. Lee, B.B., Ravindra, P., and Chan, E. 2013. Size and shape of calcium alginate beads produced by extrusion dripping. Chemical Engineering & Technology, 36. https://doi.org/10.1002/ceat.201200402
29. Lee, Y.K., and Salminen, S. 2009. Handbook of Probiotics and Prebiotics. John Wiley & Sons.
30. Liu, H., Wang, S., Cai, Y., Guo, X., Cao, Z., Zhang, Y., Liu, S., Yuan, W., Zhu, W., Zheng, Y., Xie, Z., Guo, W., and Zhou, Y. 2017. Dietary administration of Bacillus subtilis HAINUP40 enhances growth, digestive enzyme activities, innate immune responses and disease resistance of tilapia Oreochromis niloticus. Fish & Shellfish Immunology, 60, 326–333. https://doi.org/10.1016/j.fsi.2016.12.003
31. Madhusudan, N.C., Ramachandra, C.D., Udaykumar, N.D., Sharnagouda, H.D., Nagraj, N.D., and Jagjivan, R.D. 2017. Composition, characteristics, nutritional value and health benefits of donkey milk: A review. Dairy Science & Technology.
32. Mingmongkolchai, S., and Panbangred, W. 2018. Bacillus probiotics: An alternative to antibiotics for livestock production. Journal of Applied Microbiology, 124, 1334–1346. https://doi.org/10.1111/jam.13690
33. Murua, A., Todorov, S.D., Vieira, A.D., Martinez, R.C., Cencič, A., and Franco, B.D. 2013. Isolation of bacteriocinogenic Lactobacillus plantarum from donkey milk. Journal of Applied Microbiology, 114, 1793–1809. https://doi.org/10.1111/jam.12189
34. Nandi, A., Yan, L.J., Jana, C.K., and Das, N. 2019. Role of catalase in oxidative stress- and age-associated degenerative diseases. Oxidative Medicine and Cellular Longevity, 2019, 9613090. https://doi.org/10.1155/2019/9613090
35. Pan, L.X., Fang, X.J., Yu, Z., Xin, Y., Liu, X.Y., Shi, L.E., and Tang, Z.X. 2013a. Encapsulation in alginate–skim milk microspheres improves viability of Lactobacillus bulgaricus. International Journal of Food Sciences and Nutrition, 64, 380–384. https://doi.org/10.3109/09637486.2012.749841
36. Pan, L.X., Fang, X.J., Yu, Z., Xin, Y., Liu, X.Y., Shi, L.E., and Tang, Z.X. 2013b. Encapsulation in alginate–skim milk microspheres improves viability of Lactobacillus bulgaricus. International Journal of Food Sciences and Nutrition, 64, 380–384. https://doi.org/10.3109/09637486.2012.749841
37. Petrariu, O.A., Barbu, I.C., Niculescu, A.G., Constantin, M., Grigore, G.A., Cristian, R.E., Mihaescu, G., and Vrancianu, C.O. 2023. Role of probiotics in managing various human diseases. Frontiers in Microbiology, 14, 1296447. https://doi.org/10.3389/fmicb.2023.1296447
38. Pramanik, S., Venkatraman, S., and Vaidyanathan, V.K. 2023. Development of engineered probiotics with tailored functional properties. Food Science and Biotechnology, 32, 453–470. https://doi.org/10.1007/s10068-022-01156-0
39. Riaz Rajoka, M.S., Mehwish, H.M., Siddiq, M., Haobin, Z., Zhu, J., Yan, L., Shao, D., Xu, X., and Shi, J. 2017. Identification and probiotic potential of Lactobacillus rhamnosus isolated from human milk. LWT – Food Science and Technology, 84, 271–280. https://doi.org/10.1016/j.lwt.2017.05.055

40. Salvetti, E., Torriani, S. and Felis, G.E. 2012. The genus Lactobacillus: A taxonomic update. Probiotics and Antimicrobial Proteins, 4, 217–226. https://doi.org/10.1007/s12602-012-9117-8
41. Sana, S., Sheikh, A.A., Maheen, Z., Mukhtar, N., Yaqub, T., Ali, S., Aftab, M.N., and Liaqat, I. 2023. Fundamentals of Microbiology: A Laboratory Manual. Scientific Knowledge Publisher.
42. Shu, G., He, Y., Chen, L., Song, Y., Meng, J., and Chen, H. 2017. Microencapsulation of Lactobacillus acidophilus by xanthan–chitosan. Polymers, 9, 733. https://doi.org/10.3390/polym9120733
43. Siragusa, S., De Angelis, M., Di Cagno, R., Rizzello, C.G., Coda, R., and Gobbetti, M. 2007. Synthesis of γ-aminobutyric acid by lactic acid bacteria. Applied and Environmental Microbiology, 73, 7283–7290. https://doi.org/10.1128/AEM.01064-07
44. Song, M., Yun, B., Moon, J.H., Park, D.J., Lim, K., and Oh, S. 2015. Characterisation of selected Lactobacillus strains for probiotics. Korean Journal for Food Science of Animal Resources, 35, 551–556. https://doi.org/10.5851/kosfa.2015.35.4.551
45. Sorokulova, I.B., Pinchuk, I.V., Denayrolles, M., Osipova, I.G., Huang, J.M., Cutting, S.M., and Urdaci, M.C. 2008. Safety of two Bacillus probiotic strains. Digestive Diseases and Sciences, 53, 954–963. https://doi.org/10.1007/s10620-007-9930-1
46. Soto Del Rio, M.L., Andrighetto, C., Dalmasso, A., Lombardi, A., Civera, T., and Bottero, M.T. 2016. Isolation of lactic acid bacteria from donkey milk. Journal of Dairy Research, 83, 383–386. https://doi.org/10.1017/S0022029916000348
47. Sultana, K., Godward, G., Reynolds, N., Arumugaswamy, R., Peiris, P., and Kailasapathy, K. 2000. Encapsulation of probiotic bacteria with alginate–starch. International Journal of Food Microbiology, 62, 47–55. https://doi.org/10.1016/S0168-1605(00)00380-4
48. Sun, Q., Yin, S., He, Y., Cao, Y., and Jiang, C. 2023. Biomaterials and encapsulation techniques for probiotics. Nanomaterials, 13. https://doi.org/10.3390/nano13010123
49. Urdaci, M.C., Bressollier, P., and Pinchuk, I. 2004. Bacillus clausii probiotic strains: Antimicrobial and immunomodulatory activities. Journal of Clinical Gastroenterology, 38, S86–S90.
50. Vega-Sagardía, M., Rocha, J., Sáez, K., Smith, C.T., Gutiérrez-Zamorano, C., and García-Cancino, A. 2018. Encapsulation of Lactobacillus fermentum UCO-979C strain in alginate–xanthan gum. Journal of Functional Foods, 46, 504–513. https://doi.org/10.1016/j.jff.2018.05.014
51. Xu, Q., Wei, L., Chen, X., Zhu, H., Wei, J., Zhu, M., Khan, M.Z., Wang, C., and Zhang, Z. 2025. Nutritional composition and biological activities of donkey milk. Foods, 14, 2337. https://doi.org/10.3390/foods14102337
52. Zawistowska-Rojek, A., Kośmider, A., Stępień, K., and Tyski, S. 2022. Adhesion and aggregation properties of Lactobacillaceae strains. Archives of Microbiology, 204, 285. https://doi.org/10.1007/s00203-022-02877-8
53. Zheng, J., Wittouck, S., Salvetti, E., Franz, C.M., Harris, H.M., Mattarelli, P., O'Toole, P.W., Pot, B., Vandamme, P., and Walter, J. 2020. Taxonomic revision of the genus Lactobacillus. International Journal of Systematic and Evolutionary Microbiology, 70, 2782–2858. https://doi.org/10.1099/ijsem.0.004107
54. Živkov Baloš, M., Ljubojević Pelić, D., Jakšić, S., and Lazić, S. 2023. Donkey milk: Chemical composition and nutritional properties. Journal of Equine Veterinary Science, 121, 104225. https://doi.org/10.1016/j.jevs.2022.104225