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Development of probiotic microencapsulated *Limosilactobacillus reuteri* films using mucoadhesive polymers as a delivery system – strategies to improve bacterial viability

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ABSTRACT

Dysbiosis, an imbalance within the oral microbiome, is associated with several diseases, particularly periodontitis, which is characterized by a transition towards Gram-negative bacteria. Conventional treatments, including antibiotics and surgery, have limitations, which have led to an exploration of alternative methods. The application of probiotic bacteria to restore a balanced microbiome is such a minimally invasive alternative. In practice, the use of probiotic bacteria is hampered by the insufficient survival rate of the bacteria in the formulations. Using *Limosilactobacillus* strains as an example, this study addresses these challenges and discusses three concrete measures to extend the duration of the bacterial viability. First, bacterial cultures were exposed to stress inducers during cultivation, such as osmotic stress or acidic pH, to induce protective physiological responses and enhance resilience. Next, the probiotic bacteria were microencapsulated via spray-drying with Eudragit® EPO & RL30D. Besides the protective effects, the aim of microencapsulation is to ensure the gradual release of the bacteria, *i.e. Limosilactobacillus reuteri* (formerly known as *Lactobacillus reuteri)*. Finally, films comprising mucoadhesive polymers were created with the objective of prolonging the residence of bacteria in the oral cavity through mucoadhesive interactions and rendering the bacteria in a form that is suitable for application. Our research underscores the significance of cultivation conditions in improving bacterial survival in subsequent formulation steps. We confirm the efficacy of microencapsulation of *L. reuteri* through spray-drying. Its success is evidenced by the controlled release identified during the dissolution process. A suitable method for the production of mucoadhesive polymer films is described. Encountered challenges when embedding microencapsulated bacteria in polymer films are discussed and a set of conditions, including growth phase, pH, and osmotic stress, was evaluated to identify factors influencing survival. In summary, the results enhance the progress of focused measures for preserving dental health, highlighting the capability of mucoadhesive polymer films as delivery vehicles for microencapsulated probiotic bacteria.

Introduction

The oral microbiome, a dynamic ecosystem in the human oral cavity, is a complex collection of bacteria, fungi, and viruses [1]. This intricate microbial community thrives on a variety of colonization substrates, including tooth surfaces and mucosal tissues [2]. The coexistence and interactions among these microorganisms contribute to the overall health and balance of the oral environment [3,4].

Abbreviations: CFU, colony forming units; CLSM, confocal laser scanning microscope; DMSO, Dimethyl sulfoxide; EDC, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide; HPMC, Hydroxypropyl methylcellulose; MO, microorganisms; MRS, Man Rogosa Sharpe; MWCO, Molecular weight cut-off; NaCl, Sodium chloride; NHS, N-Hydroxysuccinimide; OD, optical density; PVA, polyvinyl alcohol, SEM, scanning electron microscope.

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The coexistence is a very fine balanced system [5]. Dysbiosis, an imbalance in the composition and function of the oral microbiome, has been shown to be a key factor in the development of several diseases [6]. The dysregulated microbial community triggers an inflammatory response in the host, which initiates a cascade of events leading to tissue destruction [7,8]. One notable aspect of this complexity is the shift towards Gram-negative bacteria, a phenomenon associated with the development of periodontitis [6]. Periodontitis is an irreversible pathological disease of the periodontium. The onset of this condition is marked by reversible gingivitis, which is characterized exclusively by involvement of the gingival tissues. This then progresses into periodontitis through a sequence of pathological developments and inflammation within the periodontium. Ultimately, further disease advancement results in tooth loss [9]. Several risk factors, such as smoking and diabetes, have been recognized as being causal to its incidence [10]. Antibiotics and surgical interventions are primarily used to treat periodontitis [11]. However, antibiotic therapies have been linked to negative consequences for the entire microbiome, and surgical approaches have frequently neglected the microbiome [11]. Another method for rebalancing the microbiome is through the introduction of probiotic bacteria. Probiotics are non-pathogenic microorganisms that are considered to have a beneficial effect on the health of the host [4, 12].

Spray-drying is emerging as a widely used technique for microencapsulation, known for its gentle and rapid drying process coupled with high survival rates for bacteria [13,14]. Variations in structural results can be achieved by using both two-way and three-way nozzles [15]. In two-way nozzle systems, the gas and the liquid feed are simultaneously introduced through separate channels, allowing efficient atomization and microencapsulation, in which bacteria become embedded within a protective matrix [16]. Conversely, the three-way nozzle has two inner feeds for the spraying dispersions and an outer feed for the atomization gas. At the tip of the nozzle, the convergence of the two liquids creates microencapsulation [17]. This microencapsulation strategy aims to shield bacteria and ensure controlled release and local behavior [13]. In particular, Eudragit® E and RL serve as viable options that have demonstrated efficacy in achieving the desired results at the pH levels found in the oral cavity [18]. In addition, Eudragit® RL should contribute due to its charge to mucoadhesion, enhancing the adherence of microencapsulated entities and prolonging the local residence time [19,20], which is often limited in current therapies. Eudragit® polymers are a family of methacrylate-based copolymers widely used in pharmaceutical formulations [21]. They are valued for their tunable solubility, controlled release profiles, and protective capabilities for active ingredients. Eudragit® EPO is a cationic copolymer that dissolves at low pH, often used for taste-masking and immediate release in gastric environments, while Eudragit® RL30D is a water-insoluble, yet permeable polymer with mucoadhesive properties, enabling sustained release. Both have been used in oral, buccal, and controlled drug delivery systems [19, 21].

In addition to encapsulation in a mucoadhesive polymer, the residence time of probiotic bacteria in the oral cavity can be further prolonged using mucoadhesive films [22,23]. Mucoadhesion can result from different interactions such as Van der Waals forces, hydrogen bonds, ionic interactions, or chain entanglements [24]. It is important that the negatively charged nature of mucins generates electrostatic interactions [25]. For this purpose, polymeric films composed of hydroxypropyl methylcellulose (HPMC) and polyvinyl alcohol (PVA) were prepared for embedding probiotics, exploiting the specific interaction potentials of the polymers [23]. Spray-drying as well as film formation involves drying of the system which applies stress to the microorganisms reducing the viability dramatically [26]. The formulation of microencapsulated bacteria in mucoadhesive polymer films allows to get control over residence time and release [23]. Additionally, the bacteria are formulated into a thin and flexible form that is easily applicable [26]. Advantages of such polymer films are also connected to the application as rolled objects potentially applied within periodontal pockets in the gums [27,23]. This approach is often used for edible films [26], but also showed promising results for buccal application [22]. The combination of spray coating and film formation would allow for a flexible combination of different properties for adhesion and release.

Several approaches have been developed to enhance the resistance of bacteria to drying. It has been demonstrated that different metabolic pathways are active during the various growth phases [28]. Furthermore, it has been shown that lactic acid bacteria exhibit greater resistance to external influences during the stationary phase [29]. There are various methods to apply growth-induced stress to bacteria and induce specific metabolic pathways allowing bacteria to adapt to different stressors, including osmotic stress encountered during desiccation processes. Elevated salt concentrations have been shown to induce structural changes in the cell wall of *Lactobacillus casei* ATCC 393 [30]. Similarly, *Lactobacillus delbrueckii* subsp. *lactis* responds to hyperosmotic stress with increased autolytic activity and improved survival during freeze-drying [31]. These findings illustrate how salt-induced osmotic stress can modulate physiological traits in *Lactobacilli*, including enhanced stress tolerance and viability during processing [31,32]. Adjusting the pH during cultivation represents another strategy to enhance the stress resilience of *Lactobacilli*. Growth under acidic pH conditions has been shown to improve survival during processing and increase tolerance to environmental challenges, including osmotic stress [33–35]. In this study, we used *L. reuteri*, which was previously described to be a beneficial probiotic in the treatment of periodontitis [36]. Thus, this probiotic bacterium was formulated for application into the oral cavity to allow for colonization. It was microencapsulated with Eudragit® EPO and RL30D via spray-drying for controlled release and mucoadhesion. These microencapsulated bacteria were then embedded in mucoadhesive polymer films for good application properties and prolonged retention in the oral cavity. The bacterial survival rates could further be improved by stressing the bacteria during cultivation and using the optimal growth phase for formulation.

Materials & methods

Materials

Freeze-dried *Limosilactobacillus reuteri* DSM 32759 *(L. reuteri)* was provided by Lactopia GmbH, Saarbrücken, Germany. MRS broth and MRS agar were purchased from Carl Roth GmbH, Karlsruhe, Germany. Eudragit® EPO, RL30D & S were purchased from Evonik, Essen, Germany. Hydroxypropyl methylcellulose was purchased by Shin-Etsu Chemical, Chiyoda, Japan. Polyvinyl alcohol 18–88 was purchased from Sigma Aldrich (Darmstadt, Germany). Glycerol was purchased from Caelo, Hilden, Germany. Sodium chloride was purchased from Grüssing, Filsum, Germany. Hydrochloric acid was purchased from AnalytiChem (Oberhausen, Germany).

Methods

To process the original bacterial powder of *L. reuteri* the cryoprotectant matrix was removed by resuspending them in MilliQ water and subsequent centrifugation (5000x*g*; 5 min). This was repeated three times. This material was then used as starting sample for other experiments.

Each experiment performed was conducted in triplicate, *i.e.* each experiment was carried out from the cultivation or purification of the bacteria to the final analysis.

Microbiological analysis

The viability of *L. reuteri* was assessed via the plate count method. For each replicate, 100 mg of powder, microcapsules, or film material was used. The microencapsulated bacteria were incubated in 0.9 % NaCl

solution at 37 °C for 45 min to dissolve the polymeric matrix or shell. Subsequently, the dispersion was diluted in 0.9 % NaCl and plated on MRS agar. Following incubation at 36 °C and 5 % CO_2 for 48 h, the number of colonies was determined in triplicates. The results obtained were converted to logarithmic values, and the means and standard deviations were calculated.

Cultivation of L. reuteri in liquid culture

Bacterial cultivation in liquid culture started by plating the bacteria on MRS Agar, followed by incubation at 36 °C and 5 % CO_2 for 48 h. Colonies were picked and transferred to MRS broth, undergoing incubation under aerobic conditions at 36 °C and 5 % CO_2 for a 24-hour primary culture. The 250 mL main culture (prewarmed MRS broth) was inoculated with 1 mL of preculture, and growth was monitored through measurement of OD_{600}. Following the determination of the growth curve, bacteria were cultivated until the late exponential growth phase and stationary phase for survival comparison. Subsequently, bacteria were harvested by centrifugation and incorporated into polymer films after microencapsulation.

Activation of protection mechanisms in bacteria

To enhance bacterial survival within polymer films, activation of various resistance mechanisms was pursued through two distinct test series. In each series, a primary culture was established according to the mentioned procedure.

For cultivation under pH 5 conditions, the secondary culture, MRS broth pH 5, was inoculated from the primary culture with 1 mL. The flasks were subsequently incubated at 36 °C and 5 % CO_2 until the stationary phase was attained, totaling a 16-hour incubation period.

In the salt shock treatment, the secondary culture, MRS broth pH 6.2, was inoculated with 1 mL of primary culture. After a 6.5-hour incubation at OD_{600} 0.8, bacteria were centrifuged, dispersed in MRS broth supplemented with 0.6 M NaCl, and incubated under standard conditions for a total duration of 16 h.

Upon reaching the stationary phase, bacteria were harvested through centrifugation, followed by microencapsulation achieved through spray-drying, followed by embedding in polymer films.

Microencapsulation via spray-drying

Microencapsulation of bacteria was conducted through spray-drying utilizing polymethacrylate derivatives, specifically a 1:1 mixture of Eudragit® EPO and Eudragit® RL30D, at a concentration of 10 % total polymer content. In preliminary trials, the effect of various ratios of the Eudragit composition was evaluated. The ratio that seemed to provide the most optimal microencapsulation outcomes with respect to the release characteristics was identified. The procedure was performed using a laboratory-scale Mini spray dryer (Büchi B290, Flawil, Switzerland) configured in two distinct setups. The incorporation of bacteria into a polymer matrix was facilitated using a two-way nozzle. To minimize the thermal stress on the bacteria, a three-way nozzle configuration was employed, which allows for more controlled temperature management. The polymer solutions, Eudragit® EPO and RL30D, were dispersed in water and fed into the outer feed, while the bacteria were introduced through the inner feed. The spray-drying process was optimized for reduced thermal impact by maintaining a low inlet temperature of 55 °C and an outlet temperature of 42 °C, with a system pressure of 1.5 bar. The flow rate was regulated at 1 mL/min, the rotameter was set to 60 mm, and the aspirator was operated at 100 % capacity.

Morphology analysis

The microencapsulation was evaluated using scanning electron microscopy (SEM) (EVO HD15, Zeiss, Oberkochen, Germany). The microencapsulated bacteria were attached to SEM holders via adhesive carbon plates and followed by 100 s gold sputtering using a Quorum Q150R ES sputter coater (Quorum Technologies Ltd., East Grinstead, UK), for better conductivity. The images were captured at a voltage of 5 kV and a magnification of 5 kX.

For visualization of microparticle architecture by confocal laser scanning microscopy (CLSM) the bacteria were stained with SYTO 9 (488 nm excitation, 525 nm emission). The polymer arrangement was also analyzed by fluorescence. As Eudragit® E and RL do not provide reactive groups for fluorescence labeling, Eudragit® S was used. Eudragit® S has free reactive groups that can be readily conjugated with BODIPY (561 nm excitation, 622 nm emission). This modified polymethacrylate derivative was then added as a small fraction (1:100) to the coating materials for the imaging and determination of the core shell structure.

To prepare the conjugate, Eudragit® S100 (0.349 mmol of repeating unit) was dissolved in anhydrous DMSO (5 mg/mL), followed by the addition of EDC (2 equivalents, relative to a repeating unit of Eudragit® S100) and NHS (1.1 equivalents). The mixture was stirred at 21 °C for one hour, after which BODIPY-OH (0.1 equivalent) was added to the solution. Following an overnight reaction at 21 °C, the resulting solution was subjected to dialysis (3 kDa MWCO) in a solution of DMSO and MilliQ-water (750 mL, three times) in order to remove any residual free dye and side products. Subsequently, the purified polymer was lyophilized, resulting in a yield of 75.3 %.

Polymer films incorporating L. reuteri

Polymer films were produced using an electromotive film casting device (Coatmaster 510, Erichsen, Hemer, Germany). Aqueous solutions containing 1.5 % hydroxypropyl methylcellulose (HPMC) and 1.5 % polyvinyl alcohol (PVA) (18–88) were prepared, with glycerol incorporated as a plasticizer, constituting 20 % of the total polymer mass. The solutions were sterilized via autoclaving. Microencapsulated *L. reuteri*, sourced from either freeze-dried or liquid cultures, were then integrated into the solution through gentle mixing. This mixture was uniformly spread onto a Teflon foil using a squeegee (Erichsen, Hemer, Germany) with a slit width of 1000 μm. Subsequently, the films were dried at 37 °C in a ventilated drying chamber for a duration of approximately 1.5 h.

Disintegration of microencapsulated bacteria and polymer film

The disintegration of microcapsules and polymer films were examined through incubation on a 1.5 % agarose patch maintained at 36 °C and 100 % relative humidity. In this procedure, the bacteria were applied to a polycarbonate membrane with 50 nm pore diameter and incubated for durations of 30, 60 and 120 min. The polymer film as well as the polymer shell microencapsulating *L. reuteri* disintegrate in the presence of low fluid amount originating from the agarose patch and the high humidity of the surrounding environment atmosphere. The limited fluid amount was chosen to address the conditions within the oral cavity, where even minimal quantities of liquid are readily available. Microscopic observation of disintegration was conducted using scanning electron microscopy (SEM), after complete drying following the procedure described above. The experiment was repeated 3 times.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 10.5.0, GraphPad Software, San Diego, *CA*, USA). The specific statistical tests applied, and the corresponding *p*-values are provided in the respective figure legends. For comparisons, one-way ANOVA followed by Tukey's multiple comparisons test was applied. All data are presented as mean ± standard deviation (SD). Statistical significance was defined as $p < 0.05$ and indicated as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p <$

0.001 (***), $p < 0.0001$ (****); *ns* = not significant.

Results

Survival of L. reuteri after spray-drying

Limosilactobacillus reuteri (L. reuteri) was microencapsulated via spray-drying, utilizing Eudragit® EPO and RL30D polymers. The bacteria were taken directly from the cleaned freeze-dried starting material. For these experiments, no pre-cultivation was performed. The process employed both two-way and three-way nozzles, with each demonstrating certain bacterial survival rates.

Fig. 1 shows the viability of *L. reuteri* after spray-drying using either a two-way or three-way nozzle, compared to the not microencapsulated reference.

The control group (not microencapsulated) exhibited a viability of 10.82 log(CFU/g). Spray-drying with the two-way nozzle significantly reduced viability to 9.11 log(CFU/g) ($p < 0.001$ vs. control), while the three-way nozzle preserved survival more effectively at 10.46 log(CFU/g) ($p < 0.001$ vs. two-way nozzle). The difference between the three-way nozzle and the control was not statistically significant ($p > 0.05$). These results confirm that the three-way configuration provides superior protection during spray-drying and minimizes viability loss compared to the two-way setup.

Morphology of microencapsulated L. reuteri

The intention of the process was the coating of the probiotics, encapsulating them into the polymer matrix. SEM was used to analyze the morphology (Fig. 2). Spray-drying with both two- and three-way nozzles resulted mainly in spherical objects with smooth surfaces indicating the polymer being the outer layer of the objects. This was observed for both encapsulation approaches. The different fine structure when spraying without polymer is depicted in Fig. 2C. It shows *L. reuteri* spray-dried without the addition of Eudragit® revealing round particles consisting of rod-shaped bacteria. The figure and the differences in the surface morphology indicate that the presence of the polymers led to the smooth surfaces surrounding the probiotics by a thin polymer layer.

Following the success of microencapsulation in both experimental setups and the observed high survival during spray-drying using the three-way nozzle, it was used for subsequent investigations.

Investigation of core-shell structure

In order to investigate the internal structure of the spray-dried objects, the microorganisms were stained with SYTO 9, a green-fluorescent nucleic acid dye.

Fig. 3 illustrates the formation of a core-shell structure, wherein the bacteria stained with SYTO 9 (in green) are encapsulated by Eudragit® EPO, RL30D and S conjugated with BODIPY (in red) as a dye. The particles exhibit a red shell on the exterior, while the interior displays the stained bacteria colocalized with the Eudragit® S-BODIPY (red signal), leading to the yellow color in the merged images. This red-ring surrounding the yellow color resulting from the overlaying signal from the bacteria and the polymer is a clear indication of the core-shell structure of the system.

Disintegration of microencapsulated L. reuteri

SEM micrographs in Fig. 4 illustrate the morphological changes of spray-dried microcapsules during incubation at 36 °C and 100 % relative humidity. After 30 min, the onset of surface erosion becomes apparent. At 60 and 120 min, the structural breakdown progresses, leading to increasingly flattened and fragmented particles. In some regions, rod-shaped bacteria become visible on the surface of the disintegrating matrix. Although individual bacteria cannot be conclusively identified due to overlapping matrix remnants, the observed structures are consistent with the expected morphology of *L. reuteri*. These observations support the interpretation that moisture triggers gradual capsule disintegration over time.

Growth dynamics of L. reuteri as basis for harvesting strategies

To define appropriate harvesting time points for spray-drying, the growth kinetics of *L. reuteri* were monitored by measuring optical density at 600 nm (Fig. 5). The exponential growth phase was observed between approximately 3 and 11 h, followed by a plateau from around 12 h onwards, indicating the stationary phase. A logarithmic scale of the Y-axis allows to resolve exponential bacterial growth and the transition into the stationary phase. These two physiological stages served as the basis for harvesting bacteria either during active proliferation or after growth had ceased, to assess their impact on survival during downstream processing.

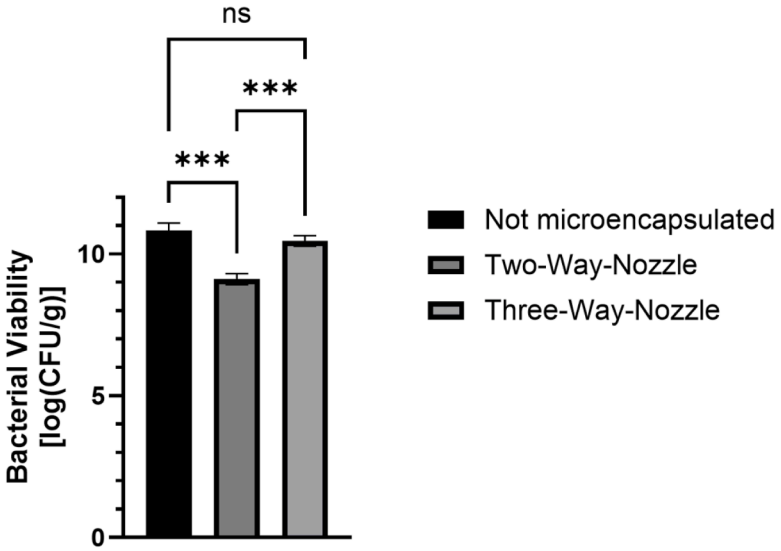

Fig. 1. Viability of *L. reuteri* after spray-drying using two-way and three-way nozzle, compared to untreated freeze-dried control. Bars represent mean ± standard deviation (n = 3). Statistical differences were determined using one-way ANOVA followed by Tukey's multiple comparisons test. ns (not significant); $p < 0.001$ (***).

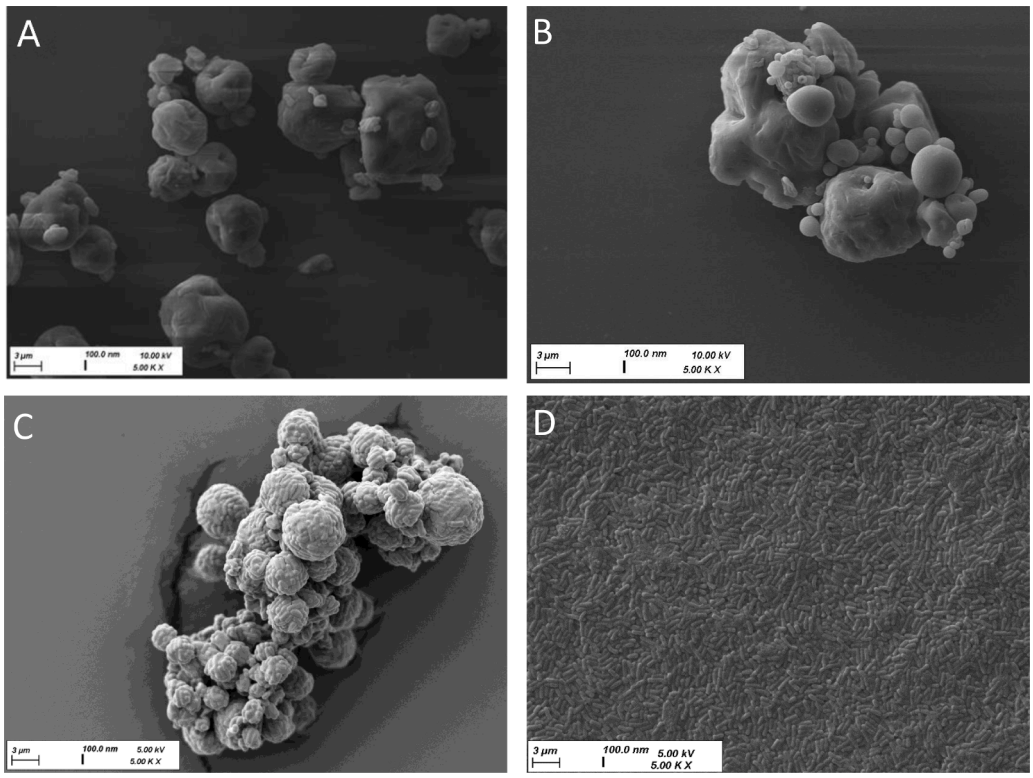

Fig. 2. Scanning electron micrographs: A: *L. reuteri* microencapsulated via two-way nozzle; B: *L. reuteri* microencapsulated via three-way nozzle. C: For comparison, *L. reuteri* was spray-dried without Eudragit® EPO and RL30D. D: SEM micrograph of a pure, non-treated *L. reuteri* sample as a reference sample.

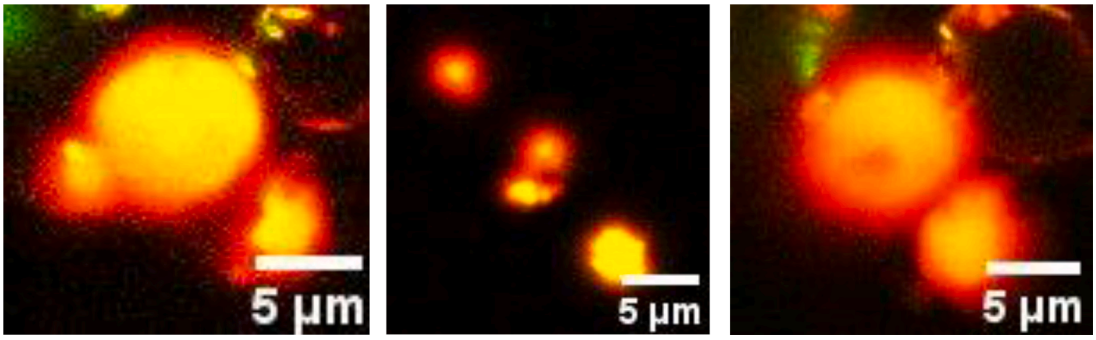

Fig. 3. Representative confocal laser scanning microscopy images show *L. reuteri* stained with SYTO 9 and microencapsulated with Eudragit® EPO, RL & S, labelled with BODIPY, and RL30D. 3 different image sections were chosen where the core-shell structure is well visible by the red color of the BODIPY label not colocalized with the SYTO 9 fluorescence of the bacteria resulting in yellow signals (overlay of red and green).

Bacterial viability after spray-drying and film embedding under different preconditions

To investigate the effect of physiological preconditioning on bacterial robustness during processing, *L. reuteri* was subjected to various stress treatments prior to microencapsulation and subsequent film embedding. The tested conditions included cultivation in acidic medium (pH 5), osmotic stress (0.6 M NaCl), combined acid and salt stress, as well as harvest during the exponential or stationary growth phase. The viability of the spray-dried starting material was included as reference value.

After spray-drying, bacterial viability varied considerably depending on the preconditioning strategy (Fig. 6). The highest survival was observed following cultivation at pH 5 (10.92 log(CFU/g)), which was significantly higher than for all other conditions, including the spray-dried starting material (10.46 log(CFU/g)). Stationary-phase cells also yielded high viability (10.54 log(CFU/g)) and did not significantly differ from the starting material ($p > 0.05$). Osmotic stress (9.79 log(CFU/g)) and combined acid and salt stress (8.72 log(CFU/g)) resulted in moderately reduced survival (each $p < 0.0001$ vs. acidic preconditioning). The lowest viability was observed when cells were harvested during exponential growth (6.50 log(CFU/g)), which was significantly lower than both the acid stress and reference group ($p < 0.0001$).

These results highlight the superior effect of acidic preconditioning and reaffirm that stationary-phase cells also offer a viable strategy for enhanced resilience [34]. In contrast, cells in the exponential phase remain particularly vulnerable to spray-drying stress.

Following film embedding, only three preconditioning strategies led to detectable bacterial survival (Fig. 7). The highest viability was again observed in acid-preconditioned cells (6.43 log(CFU/g)), which was significantly higher than both the stationary-phase (4.76 log(CFU/g)) and the salt-stressed group (4.31 log(CFU/g), each $p < 0.0001$). The difference between stationary and salt-stressed cells was not statistically significant ($p > 0.05$). All other conditions, including exponential-phase

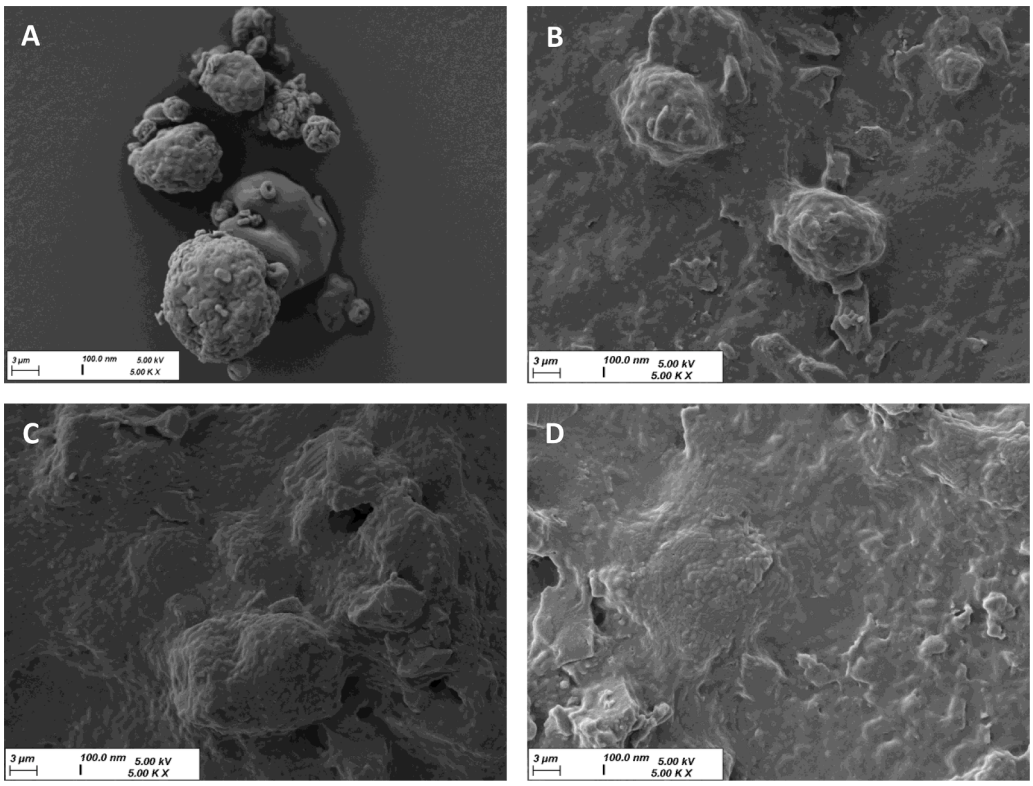

Fig. 4. Series of representative SEM micrographs taken from 3 independent experiments. The micrographs illustrate the disintegration of microencapsulated *L. reuteri* on 1.5 % agarose gel patches at 36 °C and 100 % relative humidity. Samples were imaged after different incubation times A: 0 min, B: 30 min, C: 60 min, and D: 120 min.

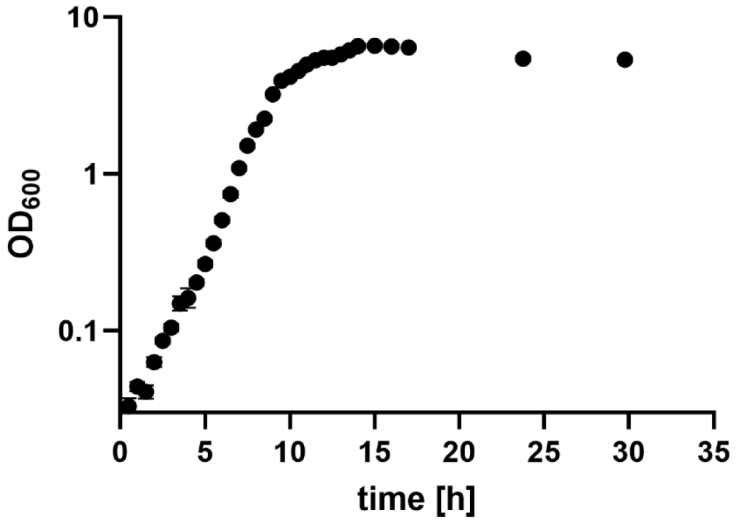

Fig. 5. Growth curve of *L. reuteri* in MRS broth. Optical density at $\lambda = 600$ nm (OD_{600}) was recorded over 30 h to determine the bacterial growth phases. Values represent mean ± SD (n = 3).

cells, the combined acid and salt stress group, and the untreated control, resulted in complete loss of viability, with no colony-forming units detected post-embedding.

In summary, cultivation at pH 5 proved to be the most effective strategy to maintain bacterial viability during both spray-drying and spray-drying plus subsequent film formation. Stationary-phase and salt-preconditioned bacteria provided limited but comparable protection, while other conditions were insufficient to ensure survival through processing. These results emphasize the importance of targeted physiological adaptation for maintaining probiotic stability in dry delivery systems.

Disintegration of polymer film containing microencapsulated L. reuteri

The release of bacteria and thus the control about availability at the site of action was a key idea of the current work differentiating the work from other formulation approaches such as lozenges. The release of bacteria from microencapsulation and polymer films was analyzed by incubating the samples on agarose patches at 36 °C and 100 % relative humidity, which was also the temperature and humidity used for the

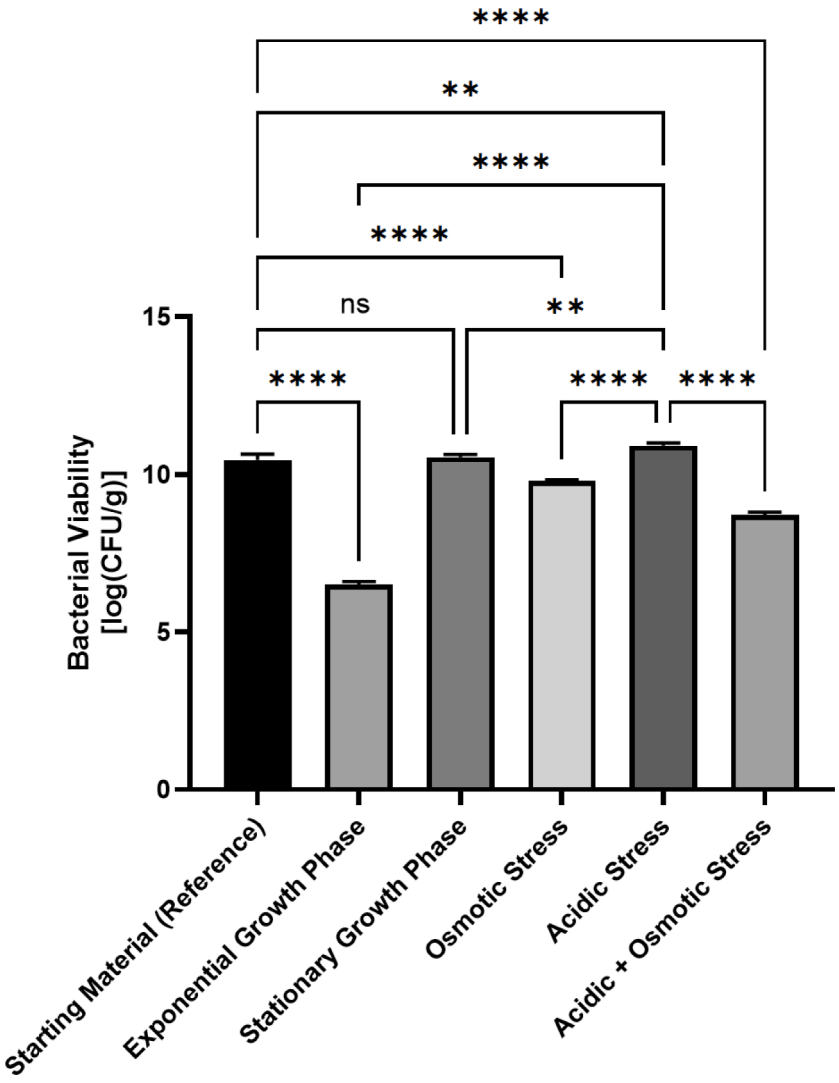

Fig. 6. Viability of *L. reuteri* after spray-drying under different preconditioning strategies. Bacteria were subjected to various physiological treatments prior to encapsulation, including cultivation at pH 5 (acid stress), 0.6 M NaCl (osmotic stress), combined acid + salt stress, or harvested during exponential or stationary growth phase. Bars represent mean values ± standard deviation (n = 3). Statistical differences were determined by one-way ANOVA followed by Tukey's multiple comparisons test. ns = not significant; $p < 0.01$ (**); $p < 0.001$ (***); $p < 0.0001$ (****).

microencapsulation disintegration. In this experiment, polymer films containing microencapsulated *L. reuteri* cultivated at pH 5 were the selected material, as it has been demonstrated that this combination exhibits the highest viability. The disintegration was evaluated by SEM analysis.

Fig. 8A depicts the microencapsulated particles embedded in the polymer film, as well as individual free, rod-shaped bacteria at a time of 0 min. After 30 min (Fig. 8B), an increase in the number of free bacteria is observed. This trend persists for 60 min, as illustrated in Fig. 8C, with an increasing number of released bacteria. By 120 min (Fig. 8D), only free, rod-shaped *L. reuteri* can be observed, and the microencapsulation structure has completely disintegrated. Therefore, it can be concluded that *L. reuteri* shall gradually be released in the oral cavity over a period of 120 min.

Discussion

The successful microencapsulation of *L. reuteri* was achieved using Eudragit® EPO and RL30D polymers. In this process, the bacteria were coated with a polymer layer for both two- and three-way nozzles, which was confirmed by SEM examination. The analysis revealed the presence of particles with a smooth and uniform surface, which can be attributed to the properties of the Eudragit® polymers (Fig. 2). The morphology of the particles within the core-shell structure can be observed by staining with BODIPY and SYTO 9 under CLSM. The shell's coloration is distinctly visible, while in the core, the SYTO 9 coloration of the bacteria is colocalized with BODIPY used for the staining of the polymer. This could be attributed to the higher intensity of BODIPY or the accumulation of some polymer inside the particles but clearly supports the successful incorporation of the microorganisms into the microparticles.

Microencapsulating *L. reuteri* did not show a strong impact on bacterial viability. The values of 10.46 log (CFU/g) were observed when utilizing the three-way nozzle and freeze-dried bacteria.

Further, the microencapsulation process resulted in a delayed release, as evidenced by the disintegration process over time of the microencapsulated bacteria on an agarose patch (Fig. 4). After 120 min, the particles were found to be completely disintegrated, with only free bacteria and polymer residues remaining. The delayed release will allow the bacteria to gradually colonize the oral mucosa and tooth enamel. When using Eudragit® EPO, the pH value of the oral cavity, which is typically around 7, is exploited [21]. This results in the swelling of the material and the indirect release of the bacteria. Eudragit® RL30D is described to also swell at pH 7 inducing a less dense polymer network and pore formation [21]. The release of the bacteria might happen along

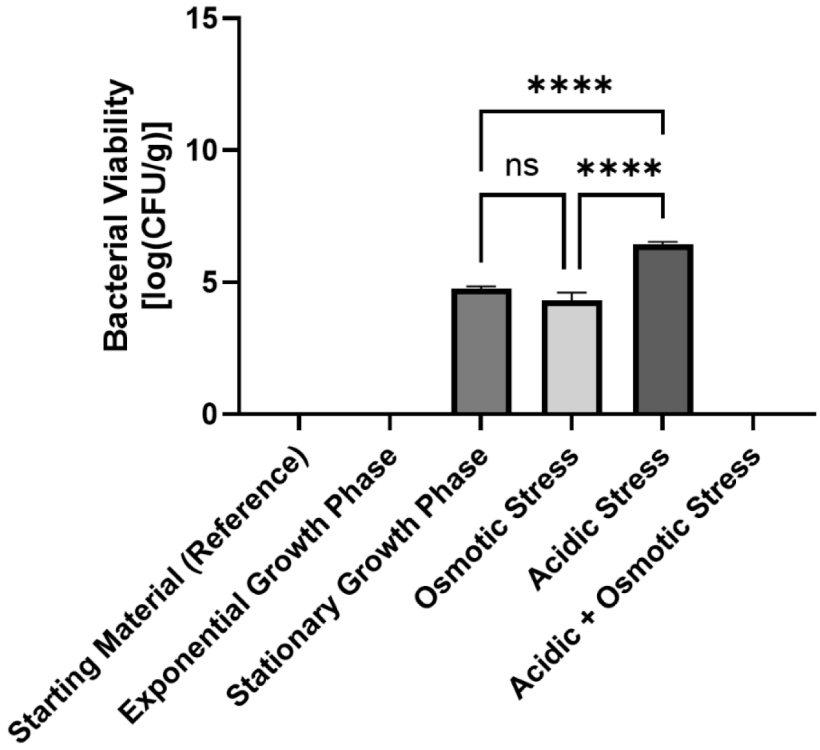

Fig. 7. Viability of *L. reuteri* after film embedding under different preconditioning strategies. Microencapsulated bacteria were incorporated into polymer films following various physiological treatments, including cultivation at pH 5 (acid stress), 0.6 M NaCl (osmotic stress), combined acid + salt stress, or harvesting during exponential or stationary growth phase. Bars represent mean values ± standard deviation (n = 3). Statistical differences were determined by one-way ANOVA followed by Tukey's multiple comparisons test. ns = not significant; $p < 0.0001$(****).

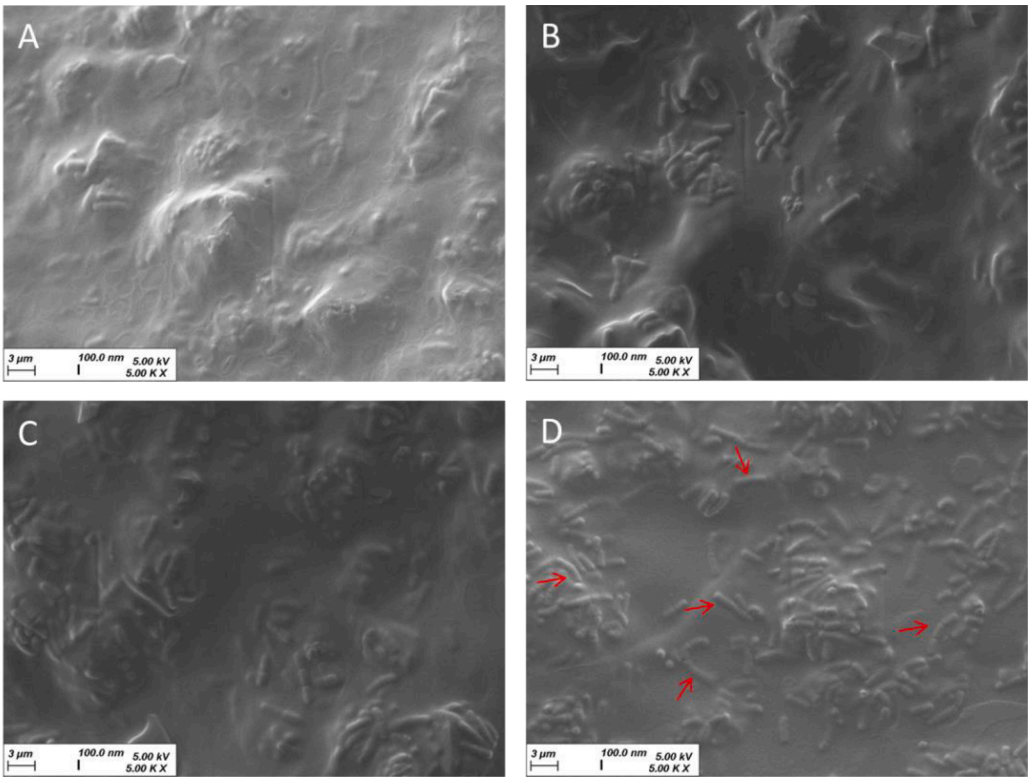

Fig. 8. Series of SEM micrographs illustrating the dissolution of polymer films (HPMC & PVA) containing microencapsulated *L. reuteri* on 1.5 % agarose gel patches at 36 °C and 100 % relative humidity. Samples were imaged after different incubation times A: 0 min, B: 30 min, C: 60 min, and D: 120 min.

those pores. Furthermore, Eudragit® RL30D is expected to exhibit delayed release and mucoadhesive properties due to the positive charge of the quaternary ammonium group, which enables it to interact with negatively charged mucins [19–21,37]. As with the particles, dissolution of the polymer films was observed over a 120-minute period. Fig. 8 illustrates that as the microcapsules undergo disintegration over time, an increasing number of bacteria were released, and the particle structures broke down. After 120 min, no residual polymer film is discernible. The SEM images demonstrate the simultaneous disintegration of the polymer film and microencapsulation, with the release of bacteria from the outset. This delayed release mechanism provides support for the colonization of the bacteria as previously described.

In contrast to the results for microencapsulation, no observable viability was detected following the introduction of encapsulated *L. reuteri* into polymer films of HPMC and PVA. This may be attributed to damage caused by earlier freeze-drying processes of the freeze-dried bacteria that were used initially [38]. In a similar system for *Lactobacillus brevis* CD2, Abruzzo et al. only adsorbed the probiotics onto the films obtained lower drying effects and thus higher viability [22]. However, we could show that the growth phase can have an impact on the survival of the bacteria. The exponential growth phase during which the bacteria were harvested and freeze-dried is also uncertain. This might also influence the subsequent viability following film casting. While spray-drying has a shorter drying time, the comparatively longer duration of 1.5 h during film casting leads to slower changes in the water concentration, which could be excessively harsh. Preliminary results indicated that an increase in drying time resulted in a corresponding increase in the mortality of bacteria. When polymer films were subjected to drying at 21 °C in the absence of forced ventilation, the process was observed to take approximately five hours, resulting in a complete loss of *L. reuteri* viability. To circumvent the damage caused by prior freeze-drying, liquid cultures were employed. A growth curve was recorded to distinguish between the exponential and stationary phases of *L. reuteri*, as these physiological states are known to influence stress tolerance during processing. Bacteria in the stationary phase generally exhibit increased resistance to environmental and technological stressors, while exponentially growing cells are more vulnerable [39]. These phase-dependent properties were considered to assess their impact on bacterial viability after microencapsulation and film embedding.

L. reuteri was microencapsulated using spray-drying at various stages, including the exponential and stationary growth phases, and subsequently embedded in polymer films. Cells harvested during exponential growth exhibited low survival after spray-drying (6.50 log CFU/g) and did not withstand the subsequent embedding step. In contrast, stationary-phase cells showed high post-drying viability (10.54 log CFU/g) and remained partially viable after film incorporation (4.76 log CFU/g) (Figs. 6 & 7). These findings highlight the critical influence of the bacterial growth phase on process resilience and align with previous reports demonstrating improved drying tolerance of stationary-phase bacteria compared to exponential-phase cells [29,40]. Our data support the use of stationary-phase cultures as a viable approach for generating probiotic film formulations with retained bacterial viability.

To further enhance bacterial robustness, two preconditioning strategies, acidic growth and osmotic adaptation, were evaluated, both individually and in combination. Osmotic stress was induced by supplementing the culture medium with 0.6 M NaCl, while acidic preconditioning was achieved by cultivation at pH 5. The latter condition is known to activate stress-related metabolic pathways and induce changes in membrane composition [34,38]. Both treatments improved survival after microencapsulation: 9.79 log CFU/g for NaCl-preconditioned cells and 10.92 log CFU/g for acid-adapted cultures, surpassing the standard cultivation reference (10.54 log CFU/g). After film embedding, acid-preconditioned bacteria maintained the highest viability (6.43 log CFU/g), whereas osmotic stress led to stronger losses (4.31 log CFU/g). Combining both stressors proved to be too severe; although spray-drying survival remained acceptable (8.72 log CFU/g), film embedding caused a total loss of viability. To the best of our knowledge, no studies have systematically investigated whether cultivation conditions directly influence bacterial resistance to the film embedding process in combination with spray-drying. While previous research has demonstrated that physiological preconditioning, such as acid stress, osmotic adaptation, or growth phase, can enhance bacterial tolerance to various technological stresses including drying, storage, and acidic environments (*e.g.*, [39]), it remains unclear whether such adaptations also improve survival during incorporation into polymer films. Our findings provide first experimental evidence that specific cultivation strategies can indeed enhance probiotic viability not only during spray-drying but also throughout the film embedding step.

This work presents a novel approach to improving oral health by developing polymer films from mucoadhesive polymers as a delivery system for microencapsulated *L. reuteri*. Unlike existing research, which primarily focuses on edible films for food packaging or gastrointestinal delivery [26,41,42], this work targets the oral cavity, addressing dysbiosis associated with conditions such as periodontitis. The study combines stress-induced bacterial resilience with microencapsulation via spray-drying using Eudragit polymers to enhance bacterial survival and ensure controlled release. The dual strategy of encapsulation and mucoadhesion significantly advances the application of probiotics for restoring the oral microbiome and offers a localized, effective alternative to conventional treatments.

Conclusion

In summary, the effectiveness of encapsulating *L. reuteri* was confirmed through multiple experimental approaches. SEM analysis revealed the presence of a continuous polymer coating surrounding the bacteria, resulting in spherical particles with smooth surfaces. These structural features correlated with maintained viability after encapsulation, indicating that the process itself does not inherently compromise bacterial survival.

A key functional advantage of the microencapsulation system was its ability to delay the release of bacteria. Upon incubation on agarose under moist conditions, a gradual disintegration of the polymer matrix was observed. After 120 min, the spray-dried objects with encapsulated bacteria lost their structure, leaving behind residual polymer with bacteria embedded. This postponed release profile supports the suitability of the formulation for controlled delivery applications, especially at mucosal sites.

Embedding the microencapsulated *L. reuteri* into polymer films composed of HPMC and PVA revealed critical vulnerability. Under standard conditions, no viable bacteria could be recovered from the films. This underscores the high stress load introduced during film casting and drying, making bacterial survival strongly dependent on prior physiological conditioning.

To improve viability during film formation, various preconditioning strategies were tested. Cultivation until the stationary phase significantly increased survival during both spray-drying and film embedding. Additionally, growth under acidic conditions (pH 5) further enhanced bacterial robustness and yielded the highest overall survival rates after both processing steps. In contrast, harvesting during the exponential growth phase, as well as applying combined acidic and osmotic stress, resulted in complete loss of viability after embedding.

These findings demonstrate that the physiological state of the bacteria at the time of processing plays a decisive role in determining their post-processing viability. While encapsulation itself offers robust protection and delayed release, successful integration into functional polymer films requires targeted adaptation of cultivation parameters. Overall, this study highlights the importance of optimizing upstream conditions—such as growth phase and medium composition—to develop effective, shelf-stable probiotic delivery systems based on microencapsulation and mucoadhesive film technologies.

Availability of supporting data

The data used and analyzed during the current study are available from the corresponding author upon reasonable request

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT-4o (OpenAI, June 2025 version) to improve the clarity, readability, and language of the manuscript. After using this tool, the authors critically reviewed, edited, and verified the content to ensure accuracy and integrity. The authors take full responsibility for the content of the published article.

CRediT authorship contribution statement

Charlotte Eckermann: Writing – original draft, Investigation, Formal analysis. **Christof J. Klein:** Writing – review & editing, Formal analysis. **Constanze Lasch:** Writing – review & editing, Investigation, Formal analysis, Conceptualization. **Sangeun Lee:** Writing – review & editing, Conceptualization. **Andriy Luzhetskyy:** Supervision, Resources, Conceptualization. **Marc Schneider:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Marc Schneider reports a relationship with Lactopia GmbH that includes: consulting or advisory and equity or stocks. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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