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Stabilization of *Lactobacillus reuteri* by encapsulation of bacterial cells through spray drying

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Abstract

Micro-organisms are often located within surface-associated multicellular aggregates known as biofilms. The human gastrointestinal tract (GI-tract) carries a microbiota that is constantly affected by extraneous influences, and hence upholding and improving the microbial balance has increased the request for probiotics. Several *Lactobacillus reuteri* strains have probiotic properties and are used in food technology. In this study the growth characteristics of *L. reuteri* was investigated using the morphology and stability of *L. reuteri* ATCC PTA 5289 after encapsulation through spray drying. The bacterial cells were assessed and visualized by Scanning Electron Microscopy (SEM) and Light Microscopy (LM) as well as spectrophotometry. *L. reuteri* ATCC PTA 5289 showed stable growth on polystyrene surfaces and adherence was also observed on aluminum surfaces. SEM images demonstrated morphological changes of the bacteria that occurred during the spray drying. After spray drying the preparation showed 15 % viable cells. The survival percentage of the spray-dried end product after 4 weeks of storage was approximately 33 % when stored at 5 °C, compared to 5 % at 25 °C.

Keywords: Bacterial survival; Encapsulation; *L. reuteri;* ATCC PTA 5289; Scanning electron microscope; Spray drying

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Introduction

Micro-organisms are often located within surface-associated multicellular aggregates that are known as biofilms. Between 500 and 1000 biofilm forming anaerobic species colonize the gastrointestinal tract (GI tract) [1] and biofilms are involved in 80 % or more of microbial infections in humans [2]. Biofilms have different properties

depending on the surrounding milieu and also on the particular biofilm generation [3]. The GI-tract contains several types of microorganism, both beneficial and harmful microbes such as Bacteroides, Shigella, Lactobacillus, Clostridium, Enterococcus and Escherichia [4]. Bacterial biofilms in the GI-tract tesselate mucus layers, mucosa, and the surfaces in the gut lumen. The majority of these bacteria are located in the large intestine, and approximately 10-100 of Lactobacillus species per ml can be found in the human stomach [5]. Since the microbiota in the GI-tract is constantly challenged by external factors, it becomes sensitized and can be accessed by harmful microbes that weakens the immune system by infecting the organism.

Probiotic bacteria are beneficial microorganisms that are made available to animals and humans through supplements and food and the demand for probiotics to sustain the microbial balance has increased over the years. The potential beneficial effects of probiotics are the production of antimicrobial agents, which attenuates the proliferation of harmful microbes, as well as immunomodulation in the GI-tract [6]. Probiotics can prevent diseases by inhibiting the pathogens, by modifying the metabolic activities of the pathogens by e.g. neutralization of toxins, exclusion/blocking of specific sites or pH changes, which affects the adhesion of the pathogen, followed by detachment or bacterial death. Probiotic bacteria are thought to be most beneficial when they are attached to specific sites on biomaterials or tissues, where they can settle and serve as a protective agent [7]. Prokaryotic organisms, such as *Lactobacillus reuteri*, are striving to attach to surfaces as a survival strategy [8]. Thus they remain in a favorable locality and cooperate and interact as a beneficial community. Formation of bacterial biofilms occurs because the film acts as a barrier and protects the bacteria from a potentially harmful environment. Biofilms comprise encapsulating exopolymeric substances (EPS), excreted by the bacteria themselves [9], forming the extracellular matrix (ECM) that embeds and interconnects the bacterial cells. Specific compounds on the cell surface are required for the adhesion of bacterial cells to solid surfaces, such as signaling molecules and proteins.

These so called microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) are integral

to the ECM and are needed for biofilm formation [10]. Examples of MSCRAMMs are fibrinogens, fibronectins and elastins, all binding to bacterial surfaces [10]. The formation of a biofilm gives the bacteria the opportunity to remain for a longer time in a nutritious environment, which leads to an increased metabolic efficiency, stability, and survival rate [10]. In order to colonize other surfaces, planktonic bacteria cells are released from the biofilm.

Lactobacillus reuteri is classified as a probiotic bacterium and is endogeneous to the GI tract, the oral cavity, and breast milk. The rod-shaped Gram-positive bacterium is categorized as a facultative anaerobe and the size range is approximately 0.5-1.6 µm [11]. The bacterial cell wall contains polysaccharides that are attached to peptidoglycans by strong phosphodiester bonds. Teichoic acids are also present in all *L. reuteri* species; bound to the cell membrane or to the cell wall. The optimal parameters to stimulate growth are temperatures between 30-40 °C, a pH between 5.0-6.2, and a 5 % CO2 atmosphere. *Lactobacillus reuteri* attaches to soluble and immobilized glycoproteins, erythrocytes, tissue sections, epithelial cells, and immobilized glycolipids [12]. *L. reuteri* species feature several beneficial properties, e.g. producing antimicrobial substances, such as Reuterin (β–hydroxypropionaldehyde, 3-HPA), a metabolite that is produced through anaerobic fermentation of glycerol [13].

Lactobacillus is used in various applications, in the polymer field, chemical industries, pharmaceuticals and food industries [14]. Important aspects need to be considered before formulating probiotics for dietary use: is it a habitual inhabitant of the GI-tract, is it resistant against the defense system in the digestive tract or what is the viability during and after storage at different temperatures [15]? There is a growing interest in including viable probiotics in products with a long-term ambient shelf life. A method for large quantity production of cultures containing high levels of viable probiotic cells in a suitable form for product applications, is highly desirable from a commercial point of view. It is expensive to produce and store freeze-dried powders or frozen concentrates of probiotic bacteria, and as a cost-effective alternative spray drying is widely used to produce large quantities of bacterial cultures. Adding to the benefits, a spray-dried powder can

be stored for prolonged periods. To produce viable cultures with spray drying requires that the microorganisms survive the exposure to the high temperatures being used and that the probiotic properties are maintained following the spray-drying process. The process may affect cellular components, including DNA, the cytoplasmic membrane, and the cell wall [16]. Since some probiotic properties are closely related to the structure of the bacterial surface, it is thus crucial to evaluate how the process affects the cells. It is well known that carbohydrates have protective effects for probiotic bacteria during drying, explained by the water-replacement hypothesis, where sugars act as water substitutes when the hydration shell of proteins and water molecules associated to polar residues in membrane phospholipids, are removed. The replacement will maintain the integrity of phospholipids bilayers at their hydrated spacing, which in turn preserves the structure of the membrane, thereby preventing damage during drying or freezing.

Protein denaturation during drying is similarly prevented by sugars that form hydrogen bonds with the proteins [16]. *L. reuteri* grows rapidly in cell culture media [8], and by forming multicellular aggregates and ECM during cultivation, *L. reuteri* may be inherently purposed (owing to encapsulation) to withstand the harsh conditions during spray drying. Previously published data focused on spray drying of microorganisms associated to cell integrity issues and viable counts is limited. *L. reuteri* was chosen for the spray drying study since this bacterium is well characterized with respect to both the probiotic properties and the behavior in different products [17].

In this study the growth, and adherence characteristics, of *L. reuteri* was investigated coupled with the morphology and stability of *L. reuteri* ATCC PTA 5289 after encapsulation through spray drying.

Material and Method

Preparation and cultivation of lyophilized L. reuteri

Approximately 1g of *L. reuteri* ATCC PTA 5289 (Batch no 1CDA009, Bio Gaia, Lund, Sweden) was dispersed in de man Rogosa Sharpe (MRS) broth (OXOID LTD, Hampshire, England). The suspension was incubated during 20 minutes on a tilt table in room temperature (RT). A dilution series in saline was prepared and 100 µl aliquots from each concentration was applied on

MRS agar (OXOID LTD, Hampshire, England). The plates were incubated anaerobically during 48 hours at 37 ºC.

Bacterial growth- and decline curve

Three colony-forming units (CFU) of L. reuteri ATCC PTA 5289 were inoculated in 30 ml MRS broth and incubated anaerobically at 37 °C during 186 hours in order to create a bacterial growth- and decline curve. A dilution series in saline was made and 100 µl aliquots from each dilution were applied on MRS agar plates and incubated anaerobically at 37 °C during 24 hours. The CFU were calculated and the concentration was determined.

Staining and detection of the bacteria

Slides were incubated with bacteria in petri dishes for 24 and 48 hours in an anaerobic environment, rinsed with Milli Q water and stained with crystal violet, 0.1 % and 0.5 %, during 30 minutes at RT. They were further rinsed in Milli Q water, left to dry in air and then investigated under the light microscope.

Quantification and estimation of a static bacterial biofilm

L. reuteri ATCC PTA 5289 suspensions (three CFU inoculated in 30 ml MRS broth) in an amount of 500 µl/well were applied in 48 well plates (Thermo Scientific NuncUpcell 48 multidish, Thermo Fisher Scientific, Roskilde in Denmark). Plates were anaerobically incubated during 48 hours at 37 °C. The optical density (OD) of the bacterial growth was then measured at 550 nm. After the incubation time the planktonic suspension was removed and fresh MRS broth was added and mixed with the adherent biofilm. A volume of 100 µl of each sample was then transformed to a 96 well plate and the absorbance was measured at 570 nm.

Preparation of L. reuteri for spray drying encapsulation

Ten CFU of *L. reuteri* ATCC PTA 5289 were added in 1L of MRS broth and incubated aerobically with magnetic stirring on a vibration plate during 48 hours at 37 ºC. The feed solution was agitated and attached to the spray drying device (BÜCHI, Mini spray dryer, B290, Essen, Germany) with the following settings; inlet temperature: 120 °C, outlet temperature: 73-74 °C, aspirator: 100 %, pump: 20 % and nozzle cleaner: 6-8. A fine dispersion of droplets is fed with hot air through the nozzle and the temperature of the droplets remains below the wet bulk temperature until the water totally evaporates.

Stability studies

Aluminum bags with 150 mg of spray dried *L. reuteri* ATCC PTA 5289 were stored at two different temperatures, 5 and 25 °C. Duplicate samples were analysed every second week by CFU quantification.

Scanning Electron Microscope (SEM)

The bacteria were washed and diluted in Milli Q water. Application of the bacteria was done on a graphite carbon plate (Agar Scientific Ltd. Cambridge, England) or aluminum stud (Agar Scientific Ltd. Cambridge, England), and placed in the SEM device (Zeiss, EVO LS 10, and Cambridge, England). The magnifications were 2000-20.000X.

Results

Bacterial growth and decline curve

A growth and decline curve (Fig 1.) for *L. reuteri* ATCC 5289 was constructed from the CFU/g values calculated from the MRS agar plates. The experiment was followed during 186 hours. The crystal violet staining of *L. reuteri* PTA 5289 after 48 hours incubation showed detailed cell morphology of the bacteria (data not shown). The average of the OD of the adherent bacterial biofilm was estimated to 0.888 (n=144). The amount of biofilm was approximately the same in all samples and replicates.

Small clusters of the bacteria were detected on the aluminum surface after 100h of incubation (Fig. 2). A huge area is shown where the bacteria cover the surface and a large quantity of single bacterial cells are attached and gathered together as clusters on the aluminum surface (Fig. 2).

Spray drying

Three experiments were performed using the spray drying technique. Spray drying was performed with bacteria from 10 CFU/g in 1L of MRS broth. The CFU/g of the *L. reuteri* ATCC PTA 5289 was calculated before and after the spray drying. The survival rate decreased with approximately one order of magnitude due to spray drying, reduced to 15 % viable cells (Table 1).

The spray drying process changed the morphology of *L. reuteri* ATCC PTA 5289. Before the spray drying the bacteria had a rod like appearance, but after spray drying the bacteria have gained a spherical formation (Fig. 3).

A stability trial from spray drying experiment 3 was performed. Duplicate samples of *L. reuteri* ATCC PTA 5289 stored at two different temperatures, 25 and 5 °C, were analyzed every second week during 24 weeks. The survival percentage calculated between the

number of bacteria before and after the spray drying was estimated to 4.2 %. The survival percentage of bacteria stored at 25 °C and 5 °C during 24 weeks showed a decrease of viability with time (Table 2 and figure 4). After 2 weeks of storage, the bacteria stored at 25 °C, had at survival rate of more than 100 % of the viable amount of cells directly after spray drying, presumably caused by the variations commonly associated with biological materials. The survival percentage of the spray dried end product after 4 weeks of storage at 5 °C was 32.70 %, compared to 4.70 % at 25 °C. After 24 weeks of storage at 25 °C the percentage of viable cells was reduced to 0.03 % compared to 2.40 % at 5 °C.

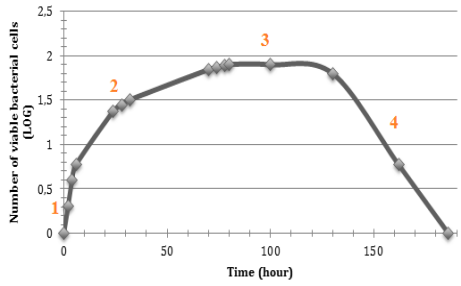

Figure 1.

L. reuteri ATCC 5289 growth and decline curve. The cultivation experiment lasted 186 hours; bacteria were incubated anaerobically at 37 °C. Concentrations of the mean values from each MRS agar plate was calculated by log CFU/g. The red figures indicate the Lag phase (1), Log phase (2), Stationary Phase (3) and the death phase (4).

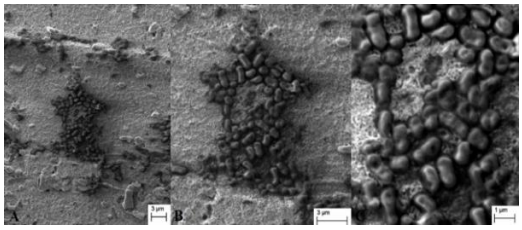

Figure 2.

L. reuteri ATCC PTA 5289 attached on aluminium after 100h of cultivation. The SEM magnifications were: 5000X (A), 10.000X (B) and 20.000X (C).

Experiment No.	Before spray drying CFU/g	After spray drying CFU/g	Survival rate (%)
1	1.10×10^{11}	0.16×10^{11}	15
2	1.50×10^{9}	0.23×10^{9}	15
3	2.40×10^{9}	0.10×10^{9}	4

Table 1.

Results from the CFU/g of the *L. reuteri* ATCC PTA 5289 calculated before and after the spray drying as well as the survival rate.

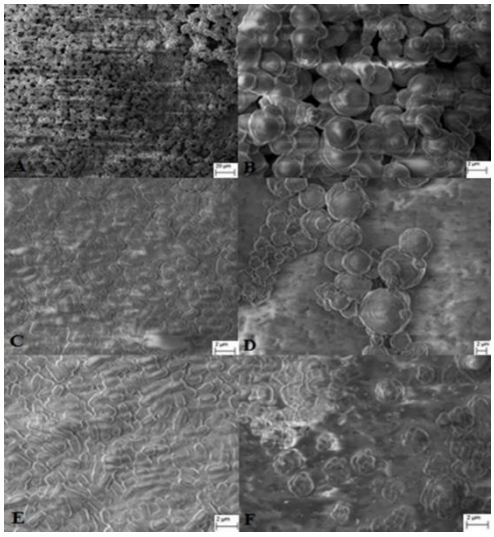

Figure 3.

L. reuteri ATCC PTA 5289 visualized by SEM before (A (1000X), C (10.000X) and E (10.000X) and after (B (10.000X), D (5000X) and F (2000X) the spray drying procedure. The bacteria were spray dried at three different occasions.

Time / Temp.	Log CFU/g											
	0	2	4	8	10	11	12	14	16	18	22	24
5 °C	8.02	NA	7.53	7.51	7.31	6.71	6.41	6.53	6.78	6.50	6.87	6.46
25 °C	8.02	8.16	6.69	5.29	6.13	6.49	4.95	4.70	4.40	4.82	4.48	4.48

NA = not analysed

Table 2.

Stability data of spray dried *L. reuteri* ATCC PTA 5289 analysed every second week during 24 weeks in two different temperatures (5 and 25 °C).

Discussion

The aim of this study was to study the growth and adherence characteristics of *L. reuteri* coupled with the morphology and stability of *L. reuteri* ATCC PTA 5289 after encapsulation through spray drying. *L. reuteri* strains are regularly applied in different products and *L. reuteri* has been described to be a robust bacterium feasible for large-scale cultivation techniques, showing high viability during production and storage.

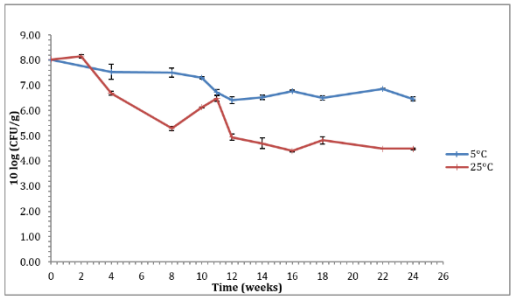

Figure 4.

Stability of spray dried *L. reuteri* ATCC PTA 5289, stored at two different temperatures, 25 and 5 °C.

The effectiveness of probiotics is strain-specific, and each strain may contribute to the host health through different mechanisms. Probiotic cultures also differ regarding the capability of withstanding the harsh conditions during food processing [17]. In order to work with the bacteria when they are growing and dividing, they should be investigated and withdrawn at the log phase to secure the optimal conditions for adhesion. According to Fig. 1 the log phase was reached at 10 hours with an absorbance range of 0.2 to 2.2. The bacteria suspension used in the cultivation was applied when the absorbance was between 0.2-0.5. Different staining techniques and dyes are used to detect and visualize bacteria, tissue sections, fungi and cells. The crystal violet (0.5 %) dye used in this study provided high contrast and defined structure of the bacteria.

The spray drying end product produced depends on many factors: the water activity (aw) of the sample, the inlet and outlet temperature, aspiration, spray gas flow, relative humidity (RH) and the concentration of the sample. For instance, a higher inlet temperature increases the outlet temperature, which reduces the relative humidity in the gas that results in a dryer product and a higher yield. The amount of the end product depends on the amount and the

saturation of the sample, which is added into to the spray-drying device. The optimal settings for the spray-drying process were defined and three experiments were performed. Different results were obtained because of the variation of the volume, storage and concentration of the samples (data not shown). For the spray drying, bacteria grown in MRS medium up to the stationary phase were used, since reduced proliferation rate, less physiological activity and increased resistance to stress seem to be most appropriate for the process [18]. Approximately 15 % of the *L. reuteri* ATCC 5289 survived the spray drying procedure and a possible optimization increasing the survival rate could be encapsulation by using polymers [19]. Polymers would protect the bacteria from the environment and thus increase the survival rate.

A study by Romano et al concerning two *L. rhamnosus* strains revealed a viability loss of about 50 % by using a chestnut extract as carrier for spray drying [16]. Another study by Jantzen et al demonstrated a survival rate of about 2 % using whey-microencapsulated *L. reuteri* DSM 20016 during spray drying and the survival rate of whey-microencapsulated *L. reuteri* in simulated gastric juices was 32% higher compared to non-encapsulated ones [18]. Due to the amount of the end product, stability and sustainability of the bacteria from only one trial was performed. Monitoring the survival percentage of the spray-dried bacteria during 24 weeks showed a decrease of viability with time, where the percentage was significantly lower at 25 °C, compared to 5 °C (Table 2 and figure 4). After 2 weeks of storage, the bacteria stored at 25 °C, had at survival rate of more than 100 % of the viable amount of cells directly after spray drying. Results with a percentage higher than 100 %, are presumably caused by the variations commonly associated with biological materials. *L. reuteri* are biologically active at higher temperatures, around 30-37 °C [11], when they have access to nutrients. However, storage of the bacteria at a temperature of 25 °C without any access to nutrients and supplements would be strenuous for the metabolic activity of the bacteria. These results indicate that it is more effective and optimal to store the spray-dried bacteria at lower temperature for longer periods. A suitable solution for increased survival of the spray-dried bacteria during storage could also be encapsulation with polymers before

spray drying. The Romano et al study showed that viable cells remained stable during a 3 month storage at 15 °C [16]. Our results fit well with the outcomes of Jantzen et al where they showed a 1 log cycle decrease during 4 weeks of storage at 4 °C [18].

Lyophilized *L. reuteri* ATCC PTA 5289 was suspended in MRS broth before additional laboratory procedures. This *L. reuteri* strain showed stable growth on polystyrene surfaces and adherence was also observed on aluminum surfaces (Fig. 2), indicating its adherence and biofilm forming capacity to these substratums. SEM results demonstrated that bacteria were more compact and were shorter in length when lyophilized cells and planktonic cells were compared (data not shown). The reason for the conformational change could be related to osmosis. The water penetrates from high to low concentrations to achieve equilibrium and the bacteria decrease in size in order to keep and protect the inner components such as the DNA during dehydration. According to Fig. 3, the *L. reuteri* ATCC PTA 5289 were morphologically changed of after spray drying. Spherical particles were formed through the spray drying process (Fig. 3B, D and F). In the spherical form the bacteria can be embedded in an EPS matrix, which acts as a protecting shell surrounding the bacteria.

Several studies have shown that probiotic organisms have difficulties to survive the acidic environments in the different parts of the stomach. Probiotic bacteria are usually colonizing epithelial surfaces in the distal ileum and colon, and they need to survive the passage through the acidic stomach and other areas in the GI-tract to operate effectively. To protect the bacteria they can be encapsulated using different methods and specific materials. Encapsulation in materials such as starch, alginate, beam gum, or xanthan with gellan gum, has been performed on different strains of *L. reuteri* by Muthukumarasamy et al [20].

The encapsulation methods that they used were extrusion and emulsification and the stability of the bacteria in different types of microcapsules was investigated. Results from the study showed that the survival rate of encapsulated bacteria was higher. Another study made by Chanramoili et al [21] showed increased viability of the bacterial cells of L. acidophilus CSCC 2400 when exposed to gastric conditions, using an encapsulation technique with calcium alginate as the protective capsule.

Environmental adaptation is affecting the survival mechanism of the bacteria, which indicates that the focus should be on the bacterial environment. Factors that always have an influence on the bacteria are intrinsic factors such as the water activity, pH of the environment, and nutritional composition. Extrinsic factors such as the packaging and storage temperature will also affect the stability and sustainability of the bacteria [22]. Different encapsulation methods and work with other bacteria and cultivation media could be included in further studies. Also approaches such as the use of various polymers, bacteria, incubation-, storage-, or processing conditions could prove useful. The effect of simulated gastric juices could be used to study the survival through the GI-tract regarding *L. reuteri* ATCC PTA 5289 or other bacteria.

In conclusion; *L. reuteri* ATCC PTA 5289 adhered to aluminum and polystyrene surfaces with a stable growth. Morphological changes of the bacteria were apparent after spray drying. Directly after spray drying the preparation 15 % of the cells were viable. The optimal temperature for storage and higher survival rate was shown to be 5 °C. To optimize the stability of spray dried bacteria, parameters such as; temperature, water activity, encapsulation methods, and pH need to be considered.

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Competing interests

Authors declare that we have no competing interests.

Authors Contributions

All authors wrote, read and approved the final manuscript.

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