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# Reuterin-producing *Limosilactobacillus reuteri*: Optimization of *in situ* reuterin production in alginate-based filmogenic solutions

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ABSTRACT

*Limosilactobacillus reuteri* produces reuterin via glycerol anaerobic fermentation. This compound has antimicrobial properties and is used for food preservation purposes. Filmogenic solutions constituted of polysaccharides and glycerol are also employed, however, reuterin synthesis in filmogenic solutions has not yet been reported. Thus, the aim of this study was to optimize the *in situ* reuterin production by *L. reuteri* in alginate- and glycerol based-filmogenic solution, evaluating the survival of reuterin-producing bacteria during fermentation. The study consisted of a completely randomized design employing two *L. reuteri* strains (DSM 20016 and DSM 17938). The filmogenic solutions were obtained using sodium alginate (20 g/L) and two independent variables were studied: glycerol (0–300 mmol/L) and initial biomass of *L. reuteri* (≅6, 7, and 8 log CFU/mL). The samples were analyzed every 24 h for 72 h of anaerobic fermentation (37 °C). Both *L. reuteri* strains confirmed the potential for reuterin production and were susceptible to the metabolite produced. The highest reuterin production was achieved using *L. reuteri* DSM 20016. The initial microbial biomass of 8 log CFU/mL and 100 mmol/L of glycerol increased the reuterin production. However, higher conversion yields from glycerol to reuterin were obtained using 50 mmol/L of substrate.

## 1. Introduction

First described by Kandler et al. (1980), *Lactobacillus reuteri* is a heterofermentative bacterium commonly found in humans, mainly in the gastrointestinal tract, vagina, and oral cavity (Hou et al., 2015). Recently, the genus *Lactobacillus* has been divided into 25 new genera according to its phylogenetic, phenotypical, and habitat specificities, and *Lactobacillus reuteri* has been reclassified as *Limosilactobacillus reuteri* (Zheng et al., 2020). This non-pathogenic bacterium is accepted by the European Food Safety Authority (EFSA, 2008) as a food supplement to improve gastrointestinal health. In addition, under anaerobic conditions and in glycerol- containing medium, *L. reuteri* strains can produce and accumulate high contents of 3-hydroxypropionaldehyde (3-HPA) through an enzymatic reaction catalyzed by glycerol dehydratase (Vollenweider and Lacroix, 2004). In 1988, the 3-HPA produced by *L. reuteri* was patented as reuterin (Dobrogosz and Lindgren, 1995) and is constantly related to the probiotic activity of this microorganism (Mu et al., 2018).

In fact, reuterin is a dynamic system, also named as HPA system, which contains 3-HPA, its hydrated form 1,1,3-trihydroxypropane, and its dimer 2-(2-hydroxyethyl)-4-hydroxy-1,3-dioxane (Vollenweider et al., 2003). However, it has recently been suggested to include acrolein in the reuterin system since the 3-HPA in an aqueous solution can suffer spontaneous dehydration to acrolein (Engels et al., 2016). The reuterin system has several antimicrobial characteristics, such as antifungal activity (Schmidt et al., 2018; Vimont et al., 2019), broad spectrum of activity against Gram-positive and negative bacteria (Ávila et al., 2014; Montiel et al., 2014; Langa et al., 2018; Asare et al., 2020), and antagonistic effect against some protozoa (Vollenweider and Lacroix, 2004). The exact mechanism of reuterin inhibition against microorganisms is not fully understood. However, Talarico and Dobrogosz (1989) postulated that reuterin can compromise the DNA synthesis by inhibiting ribonucleotide reductase. Schaefer et al. (2010) then suggested that reuterin may cause depletion of thiol groups in glutathione, proteins, and enzymes, inducing cells to oxidative stress that can result in cell death.

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Both reuterin and filmogenic solutions have also been associated with prolonged shelf life of foods. Reuterin is used to preserve foods by the inhibition of pathogenic and spoilage microorganisms (Angiolillo et al., 2017; Langa et al., 2018; Ortiz-Rivera et al., 2017), while an increasing number of studies have described the production of polysaccharide-based filmogenic solutions with or without microbial cells as edible films and coatings for foodstuff (Salinas-Roca et al., 2016; Guerreiro et al., 2017; Rodrigues et al., 2018). Filmogenic solutions are also used as a vehicle to encapsulate microbial cells and/or drugs, improving the stability of encapsulated agents under adverse and storage conditions (Rodrigues et al., 2017, 2020; Uyen et al., 2020).

Sodium alginate is widely used in the production of filmogenic solutions especially due to its colloidal properties. It is a linear heteropolysaccharide constituted of D-mannuronic acid residues united by β-(1→4) bonds and L-guluronic acid residues united by α-(1→4) bonds (Ching et al., 2017). This polysaccharide is considered as safe for consumption in the European Union (E 401 and E 404) and the United States (GRAS, 21 CFR 184.1187 and GRAS, 21 CFR 184.1724), and is used as a functional ingredient in foods (Qin et al., 2018).

Glycerol is also present in the composition of alginate-based filmogenic solutions (Shigematsu et al., 2018; Rodrigues et al., 2018). It can modify mechanical and permeability properties in the obtained solutions (Paixão et al., 2019). Although glycerol can be metabolized by *L. reuteri* for reuterin production under suitable conditions (Vollenweider and Lacroix, 2004), in filmogenic solutions this approach was not very studied. Malmo et al. (2013) reported the reuterin production by *L. reuteri* DSM 17938 coated in alginate-chitosan beads obtained by spray-drying. However, the *in situ* synthesis of this antimicrobial compound in filmogenic solutions has not yet been reported.

Thus, the aim of this study was to optimize the *in situ* reuterin production by *Limosilactobacillus reuteri* strains DSM 20016 and DSM 17938 in a filmogenic solution using alginate and glycerol, evaluating the cell viability of the reuterin-producing bacteria during fermentation.

## 2. Material and methods

### 2.1. Material

*L. reuteri* strains DSM 20016 and DSM 17938 were obtained from Coleção de Cultura Tropical (Fundação André Tosello) under the number CCT 3433 and isolated from PROVANCE® (Aché Laboratórios Farmacêuticos S.A.), respectively. To produce the filmogenic solutions, high viscosity sodium alginate (Dinâmica Química Contemporânea Ltda, Diadema, SP, Brazil) and glycerol ≥99.5% (Anidrol Produtos para Laboratórios Ltda., Diadema, SP, Brazil) were used. Bacteriological peptone and yeast extract were purchased from HiMedia (Mumbai India). Both de Man, Rogosa and Sharpe broth and agar were obtained from Merck (Darmstadt, Germany). The sodium chloride, tryptophan, sodium citrate and ethanol 95% were acquired from Dinâmica Química Contemporânea Ltda (Diadema, SP, Brazil). Acrolein standard was obtained from Riedel-de Haën (Seelze, Hannover, Germany).

### 2.2. Microbial growth conditions

The stock cultures of *L. reuteri* were kept frozen at −18 °C in a medium of glycerol (130 g/L), bacteriological peptone (4.3 g/L), yeast extract (2.6 g/L), and NaCl (4.3 g/L), and then were activated in de Man, Rogosa and Sharpe sterile broth. The cultures were incubated at 37 °C for 24 h under aerobic static conditions. Subsequently, *L. reuteri* biomass was obtained by centrifugation at 9800×*g* for 10 min (Sorvall Legend XTR, Thermo Scientific™, Germany) and used in the production of filmogenic solutions.

### 2.3. Optimization and determination of *in situ* reuterin production by *L. reuteri* in filmogenic solutions

The filmogenic solutions were prepared using sodium alginate (20 g/L) and different glycerol concentrations. The contents of sodium alginate and glycerol were homogenized in distilled water using a digital disperser (Ultra-turrax, IKA T25, Brazil) at 710×*g* for complete dissolution. The solutions were subjected to the vertical autoclave sterilization process (AV-SD 137, Phoenix, Brazil) at 121 °C for 15 min. After cooling to 42 °C, *L. reuteri* was inoculated to reach the desired proportions.

The study to optimize the reuterin production was conducted by a completely randomized design and both *L. reuteri* strains (DSM 20016 or DSM 17938) were tested separately. Two independent variables were tested: the glycerol concentration (0, 25, 50, 100, 200, and 300 mmol/L) and the initial biomass of *L. reuteri* (approximately 6, 7, and 8 log CFU/mL). The samples were analyzed at 0 h and after 24, 48, and 72 h of anaerobic fermentation at 37 °C. The anaerobic conditions were achieved by insufflating nitrogen inside in the sealed flasks of 100 mL containing the reactional medium, whose were purged with nitrogen gas for 7 min to ensure anaerobic conditions. The encoding of the treatments is in Table 1.

The reuterin quantification in filmogenic solutions was carried out indirectly according to the colorimetric method proposed by Circle et al. (1945), with adaptations. Initially, 1320 μL of the samples was homogenized with 300 μL of a 0.1 M tryptophan solution (dissolved in 0.05 M HCl) and 600 μL of ethanol (95%). The samples were diluted in sodium citrate (20 g/L) and incubated at 40 °C for 50 min. Then, the absorbances were measured by spectrophotometry (DU 640, Beckman Coulter, CA, USA) at 560 nm. The reuterin content was determined by comparing the absorbance of the samples with an acrolein standard curve previously constructed in the range from 2 to 100 mmol/L, assuming that 1 M of dehydrated reuterin corresponded to 1 M of acrolein.

### 2.4. *L. reuteri* cell viability

In order to relate the *L. reuteri* viability with the reuterin production, the number of *L. reuteri* viable cells present in the filmogenic solutions was determined before (0 h) and after fermentation at 37 °C (24, 48, and 72 h). Thus, 1 mL of each assay solution was diluted in a sterile peptone solution (1 g/L) followed by pour plating in de Man, Rogosa, and Sharpe agar. The samples were incubated at 37 °C for up to 72 h under aerobic conditions. Posteriorly, the *L. reuteri* viability was estimated by counting of the number of colony-forming units (CFU/mL).

### 2.5. Statistical analysis

All samplings were made in genuine triplicate. The data were analyzed with an assumption of a normal distribution and subjected to ANOVA and the means were compared by the Tukey test using the Statistica 10 software (Tulsa, OK, USA). The *t*-test was applied when the

**Table 1**
Distribution and encoding samples of optimization study of *in situ* reuterin production in filmogenic solutions.

| glycerol (mmol/L) | *L. reuteri* DSM 20016 (≅ log CFU/mL) | | | *L. reuteri* DSM 17938 (≅ log CFU/mL) | | |
|---|---|---|---|---|---|---|
| | 6 | 7 | 8 | 6 | 7 | 8 |
| **0** | A60 | A70 | A80 | D60 | D70 | D80 |
| **25** | A61 | A71 | A81 | D61 | D71 | D81 |
| **50** | A62 | A72 | A82 | D62 | D72 | D82 |
| **100** | A63 | A73 | A83 | D63 | D73 | D83 |
| **200** | A64 | A74 | A84 | D64 | D74 | D84 |
| **300** | A65 | A75 | A85 | D65 | D75 | D85 |

comparison of means between two sample groups was required. The results were considered significant at $p < 0.05$.

## 3. Results and discussion

### 3.1. Optimization of *in situ* reuterin production in filmogenic solutions

Figs. 1 and 2 present the results obtained from the optimization study of the *in situ* reuterin production by *L. reuteri* DSM 20016 and *L. reuteri* DSM 17938, respectively. The highest reuterin production in the filmogenic solutions was 68.39 mmol/L using *L. reuteri* DSM 20016 in the fermentation process, while the *L. reuteri* DSM 17938 strain produced a maximum of 30.00 mmol/L. Thus, it indicates that different *L. reuteri* strains have different reuterin production capacities through the bioconversion of glycerol. This difference can occur due to particularities in the gene expression, cell age, and the presence of heterologous cells (Spinler et al., 2008; Ortiz-Rivera et al., 2017). Thus, besides the choice of *L. reuteri* strain, both the preservation and maintenance of microbial cultures require careful attention to ensure that recovered cultures works as the originals.

The effects of the glycerol concentration on the reuterin production were tested by varying the substrate concentration from 0 to 300 mmol/L in the filmogenic solutions. The production of the antimicrobial compound was not detected during the periods analyzed in the controls (samples without glycerol) and in D61, D62, and D71. In general, higher bioconversion rates from glycerol to reuterin were obtained using 50 and 100 mmol/L of glycerol. The highest reuterin production was obtained using 100 mmol/L of glycerol in A83 ($p < 0.05$), in which the conversion yields from glycerol to reuterin were directly calculated and estimated at 68.39, 68.02, and 66.17% after 24, 48, and 72 h of anaerobic fermentation, respectively. Even higher yields were obtained in A82 (83.7 and 92.32% after 48 and 72 h of fermentation, respectively), the reuterin content produced under these conditions was about 1.5 folds lower than A83. Glycerol concentrations below 50 mmol/L (25 mmol/L) or above 100 mmol/L (200 and 300 mmol/L) were not considered effective in the process. Although the reuterin production was detected in fimogenic solutions prepared with 200 mmol/L of glycerol in A74 and A84, which are close to the highest amounts obtained using 100 mmol/L of glycerol, the bioconversion rates were lower than A83 (32.28 and 30.49%, respectively). In addition, they were only achieved after 48 and 72 h. Similar results were reported by Doleyres et al. (2005) using *L. reuteri* ATCC 53608. The authors observed a decrease in the conversion rate from glycerol to reuterin at increased glycerol concentrations (84, 82, 77, and 62% for 200, 250, 300, and 400 mmol/L of glycerol, respectively). The use of high glycerol concentrations during catalysis can inactivate the glycerol dehydratase, which is responsible for the production of electron acceptors in the fermentation of glycerol and the consequent reuterin production (Liu and Yu, 2015).

The fermentation time (24, 48, and 72 h) did not have a significant effect ($p > 0.05$) considering the highest reuterin rates (A83), showing a total reduction of 2.22 mmol/L from 24 to 72 h of fermentation. Martín-Cabrejas et al. (2017) reported the *in situ* reuterin production in cheeses supplemented with glycerol (100 up to 500 mmol/L) and *Lactobacillus reuteri* INIA P572 between 4.42 and 4.71 μmol/g on the first day of storage. However, the authors emphasized that the increase in the storage time resulted in a decrease in the reuterin concentration, reaching less than 0.6 μmol/g after 30 days. The decrease in reuterin content may be due to its interaction with the cellular material and/or free amino groups in the medium. Thus, prolonged incubation or storage can result in a decrease in the free metabolite concentration (Doleyres et al., 2005). In addition, the reuterin production can be interrupted due to its accumulation in the medium, resulting in cell death in *L. reuteri* caused by either the inhibition of ribonucleotide reductase, which compromises the DNA synthesis (Talarico and Dobrogosz, 1989), or the interaction and modification of thiol groups in active peptide structures, such as glutathione, thioredoxin, and glutaredoxin, which can induce cells to oxidative stress (Doleyres et al., 2005; Schaefer et al., 2010).

The effect of the initial biomass of *L. reuteri* on the bioconversion from glycerol to reuterin was tested at approximately 6, 7, and 8 log CFU/mL. The increase in the *L. reuteri* biomass from 7 to 8 log CFU/mL increased the reuterin production from 17.52 (A73) to 68.38 mmol/L (A83) and from 7.77 (D73) to 30.00 (D83) mmol/L after 24 h of anaerobic fermentation using *L. reuteri* DSM 20016 and *L. reuteri* DSM 17938, respectively. Besides being associated with an increase in the reuterin production, it was also observed that the increase in the initial biomass of *L. reuteri* may be related to the decrease in the fermentation

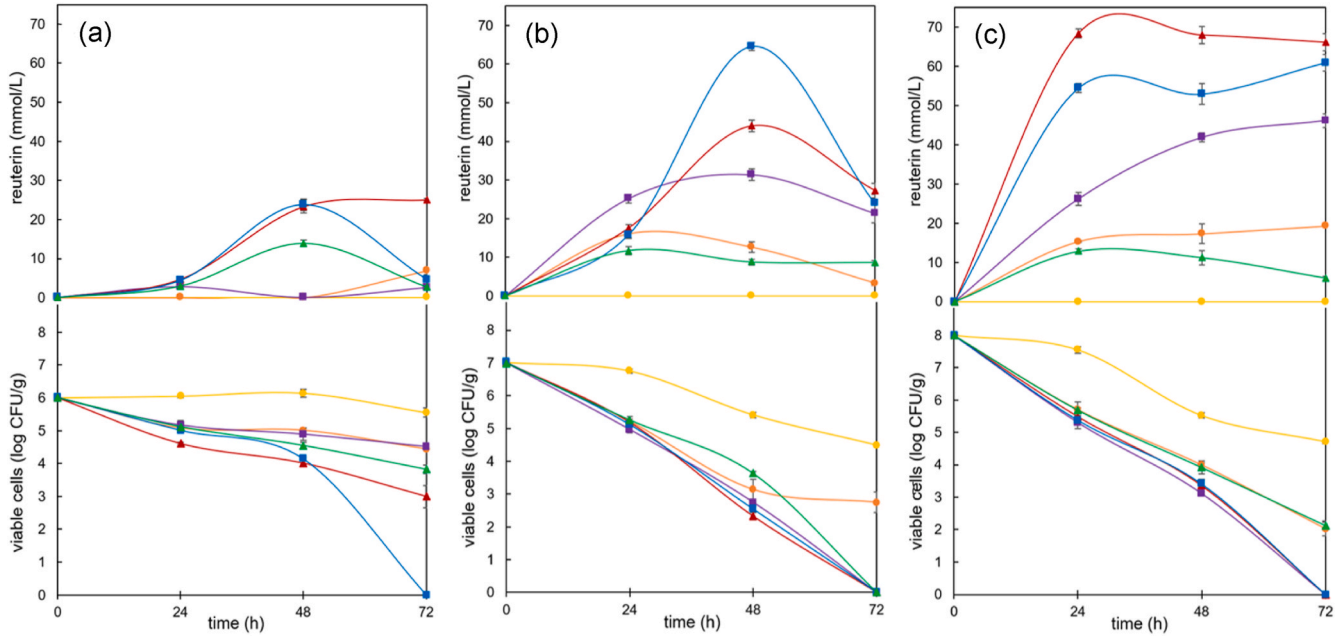


**Fig. 1.** Reuterin production and *L. reuteri* DSM 20016 cell viability according to initial biomass concentration at 6 **(a)**, 7 **(b)** and 8 log CFU/mL **(c)** at 0 and after 24, 48 and 72 h of anaerobic fermentation (37 °C), without (● – A60, A70, A80) and with glycerol at 25 (● – A61, A71, A81), 50 (■ – A62, A72, A82), 100 (▲ – A63, A73, A83), 200 (■ – A64, A74, A84) and 300 mmol/L (▲ – A65, A75, A85). *Each data point represents the mean of assays performed in triplicate.

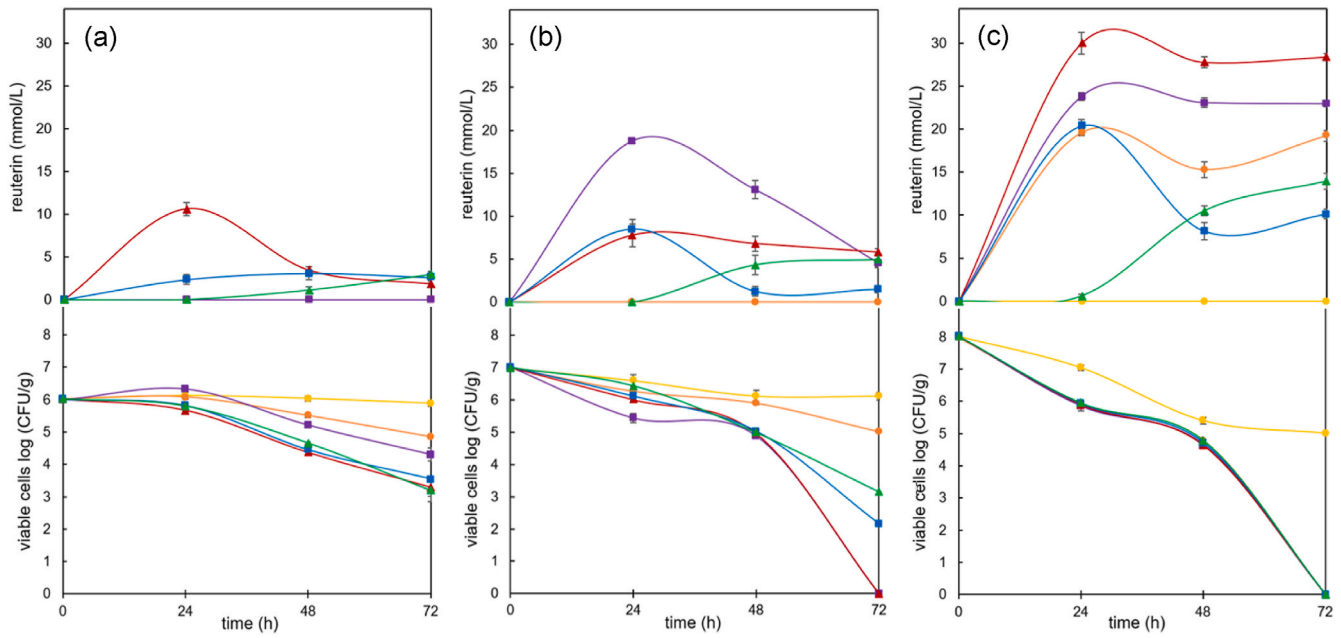


**Fig. 2.** Reuterin production and *L. reuteri* DSM 17938 cell viability according to initial biomass concentration at 6 **(a)**, 7 **(b)** and 8 log CFU/mL **(c)** at 0 and after 24, 48 and 72 h of anaerobic fermentation (37 °C), without (● – D60, D70, D80) and with glycerol at 25 (● – D61, D71, D81), 50 (■ – D62, D72, D82), 100 (▲ – D63, D73, D83), 200 (■ – D64, D74, D84) and 300 mmol/L (▲ – D65, D75, D85). *Each data point represents the mean of assays performed in triplicate.

time to obtain the higher productions of reuterin. In general, the use of the initial biomass concentration of *L. reuteri* at approximately 8 log CFU/mL coincided with the highest production of the reuterin content (A83) immediately after 24 h of fermentation (68.30 mmol/L), with no significant difference ($p > 0.05$) regarding the increase in the fermentation time to 48 and 72 h. In contrast, the initial biomass of *L. reuteri* at 7 log CFU/mL led to a slower biosynthesis from glycerol to reuterin, mainly in A73 and A74, in which the increase in the fermentation time to 48 h resulted in an increase in the reuterin production from 17.52 to 43.94 mmol/L (A73) and from 15.76 to 64.56 mmol/L (A74), representing 2.5- and 4-fold higher metabolite content, respectively. A similar behavior was reported by Doleyres et al. (2005) in a study to optimize the production of 3-HPA using a two-step fermentation process with *L. reuteri* ATCC 53608. In this study, the increase from $7 \times 10^8$ to $1.6 \times 10^{10}$ CFU/mL in the initial inoculum led to an increase from 26 mmol/L to 160 mmol/L in the production of 3-HPA, besides decreasing the incubation time from 2 h to 45 min. Ortiz-Rivera et al. (2017) have recently reported the *in situ* reuterin production in fermented milk using the initial inoculum of *L. reuteri* ATCC 52608 at approximately $5 \times 10^9$ CFU/mL and 200 mmol/L of glycerol. After fermentation, the highest reuterin content was 33.97 mmol/L. However, the authors emphasized that the production was 107.5 mmol/L in aqueous solution, suggesting that the physical and chemical changes of the reaction medium possibly influences the metabolite production, either by the presence of cofactors that favored the conversion from glycerol to reuterin or interferences in the glycerol diffusion reducing its availability to *L. reuteri* cells.

### 3.2. Survival of Limosilactobacillus reuteri in filmogenic solutions

The susceptibility of *L. reuteri* DSM 20016 and *L. reuteri* DSM 17938 to the *in situ*-produced reuterin in filmogenic solutions after anaerobic fermentation at 24, 48, and 72 h was tested. In general, the increase in the fermentation time and the consequent increase in the time of exposure to reuterin significantly decreased ($p < 0.05$) the number of viable *L. reuteri* cells present in the filmogenic solutions as compared to the controls (samples without glycerol) (Figs. 1 and 2). Martín-Cabrejas et al. (2017) optimized the reuterin production in cheese by *L. reuteri* INIA P572 initially inoculated at more than 6.3 log CFU/g. The authors reported a gradual decrease in the cell viability during 30 days of cheese ripening, reaching cell counts lower than 4.5 log CFU/g in samples with 100–500 mmol/L of glycerol, while the control (without glycerol) kept a viable cell count of 6.34 log CFU/g after the same storage time. However, the highest reuterin concentrations present in these cheeses were between 4.42 and 4.71 μmol/g, contrasting with the highest results obtained in the present study.

The use of the initial biomass of *L. reuteri* at approximately 7 and 8 log CFU/mL, usually associated with a high production of reuterin content, showed a sharp decrease in the bacterial cell viability during the bioconversion of glycerol. After 72 h of anaerobic fermentation, no viable *L. reuteri* cells were detected by the standard pour plate method employed in A72, A73, A74, A75, A82, A83, A84, D72, D73, D81, D82, D83, D84, and D85, while a low number of viable cells was detected in A71, A81, A85, D74, and D75 (2.75, 2.02, 2.13, 2.17, and 3.15 log CFU/mL, respectively). Although the cellular survival of *L. reuteri* DSM 20016 and *L. reuteri* DSM 17938 during fermentation showed a similar behavior, *L. reuteri* DSM 20016 produced higher reuterin contents (Fig. 1), suggesting that this specific strain may have greater resistance to the *in situ* reuterin produced.

In previous studies, *L. reuteri* DSM 20016 and SD 2112 showed resistance to reuterin, reaching ranges between 30–50 mmol/L and 60–120 mmol/L for the minimum inhibitory concentration and minimum bactericidal concentration, respectively (Cleusix et al., 2007). On the other hand, *L. reuteri* ATCC 53608 viable cells were not detected in the presence of 8.5 mmol/L of reuterin (Ortiz-Rivera et al., 2017). In the present study, it was observed that in filmogenic solutions with a reuterin content ranging from 7.77 to 68.39 mmol/L, the *L. reuteri* viability cell decreased rapidly after 48 h and no viable cells were detected after 72 h at 37 °C (Figs. 1 and 2).

Langa et al. (2013) reported the *in situ* reuterin production by *L. reuteri* INIA P579 in dairy products supplemented with 50 mmol/L of glycerol. In cheeses initially inoculated at 5.85 log CFU/mL, *L. reuteri* presented a decrease in the number of viable cells (>3 log CFU/g) during 30 days of storage at 12 °C, in which the highest reuterin content estimated was 5.52 mmol/L. In parallel, the same authors reported the *in situ* reuterin production in yogurt, which did not show a significant decrease in the *L. reuteri* viability during 28 days of storage, however, in

this case the storage temperature was 6 °C.

The temperature of the medium during the conversion from glycerol to reuterin may be directly related to the decrease in the *L. reuteri* cell viability. Doleyres et al. (2005) reported that reuterin can be less reactive at low temperatures, maintaining the metabolite yield or even the bacterial cell viability under these conditions. The authors reported that the use of temperatures at 5 and 15 °C during the conversion from glycerol to reuterin did not cause loss in the cell viability of *L. reuteri* ATCC 53608 and no viable cells were detected at 30 and 37 °C after 1.5 and 2 h of fermentation. However, high reuterin productions were obtained from 5 to 37 °C, demonstrating the wide activity range of the glycerol dehydratase. du Toit et al. (2011) emphasized that high temperatures increase the spontaneous non-enzymatic dehydration from 3-HPA to acrolein. This dehydration process may be related to the lower reuterin reactivity suggested by Doleyres et al. (2005). Thus, different temperatures can be adopted in the fermentation process according to the application purpose. Therefore, the fermentation temperature (37 °C) used in the present study may be associated with the increase in the reuterin reactivity or even with greater cellular sensitivity of *L. reuteri* strains as compared to the metabolite produced in filmogenic solutions, especially at temperatures close to the ideal growth temperature of the microorganisms used (Doleyres et al., 2005).

As far as we know, this is the first report on reuterin production in filmogenic solution. Furthermore, the findings of present study establish conditions for *in situ* reuterin production in filmogenic solutions constituted of alginate or another similar natural hydrocolloid. Enabling its application for different purposes, such as, edible coatings for extend the shelf life of foods and/or support material for delivery of chemical or biological compounds.

## 4. Conclusion

This study confirmed the potential of *L. reuteri* DSM 20016 and *L. reuteri* DSM 17938 for the *in situ* reuterin production in alginate-based filmogenic solutions using glycerol as substrate. Both *L. reuteri* strains studied were susceptible to reuterin produced, being that in the presence of higher reuterin concentrations an evident decrease in the cell viability of *L. reuteri* was detected. The highest reuterin production was obtained using *L. reuteri* DSM 20016. Furthermore, the use of the initial microbial biomass at 8 log CFU/mL was related to an increase in the reuterin production and a decrease in the fermentation time to obtain a higher metabolite concentration. In parallel, the use of 100 mmol/L of glycerol also improved the reuterin production, but even though approximately 1.5 folds lower, higher conversion yields from glycerol to reuterin were obtained using 50 mmol/L of the substrate.

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## CRediT authorship contribution statement

**F.J. Rodrigues:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **M.F. Cedran:** Investigation, Formal analysis, Writing – review & editing. **J.L. Bicas:** Resources, Writing – review & editing. **H.H. Sato:** Resources, Writing – review & editing, Supervision.

## Declaration of competing interest

The authors 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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