Disclosing Lactobacillus delbrueckii subsp. bulgaricus intraspecific diversity in exopolysaccharides production

# Disclosing *Lactobacillus delbrueckii* subsp. *bulgaricus* intraspecific diversity in exopolysaccharides production

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**Abstract**

Exopolysaccharides production by 3 ropy strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* of dairy origin was evaluated in synthetic medium by combining different approaches: impedometric measurements, fluorescent microscopy and flow cytometry analyses. The evaluation of ΔE by impedometric measurement ($E\%_{max}$-$E\%_{40h}$) allowed the detection of EPS production in synthetic medium, but the differences in EPS production kinetic was highlighted by flow cytometry analysis and fluorescent microcopy. This approach enabled us to unravel the diversity in EPS synthesis and release into the laboratory medium during the growth of the strains. Our results showed that the maximum EPS production occurred after 8 h of incubation, when cells were in late exponential growth phase. Furthermore, flow cytometry analysis revealed that only part of the cell population could be identified as EPS producer or as EPS-bounded cell. Therefore, the combined approach used, allowed us to define at the same time the kinetics of EPS production and release by three strains belonging to the same species and, highlight that the production of EPS depends also on the number of EPS-producing cells within the same population.. This approach could be useful for the selection of strains to be used as starter cultures in dairy products where EPS production is considered an important feature.

Keywords: Exopolysaccharides, flow cytometry, *Lactobacillus delbrueckii* subsp. *bulgaricus,* impedometric analysis, fluorescence microscopy

## 1. Introduction

The ability to produce extracellular polysaccharides (EPS) is widespread among lactic acid bacteria (LAB). Although their production may be high-energy-requiring for bacteria, benefits could be higher than costs, as they might be involved in improving growth and survival of bacteria under stress conditions (Zhou et al., 2019).

Furthermore, until today EPS have gained particular attention from food industries due to their ability to increase physical properties of food products by influencing viscosity, syneresis and sensory profiles, and prolonging shelf life (Lynch et al., 2018; Zeidan et al., 2017; Zhou et al., 2019). Besides these technological advantages, the EPS produced by LAB are also associated to various biological properties, such as prebiotic, antioxidant, anti-inflammatory, cholesterol lowering capacities, and other health benefits particularly related to their beneficial effect in the gastrointestinal tract (Guérin et al., 2020; Oerlemans et al., 2021)

EPS from LAB are classified according to their chemical composition in: homoexopolysaccharides (HoPS), when made up of the same monosaccharide, and heteropolysaccharides (HePS), if different monosaccharides are present in the chain. This differences in structure is only a first look into the complex variability that could be found among EPS from LAB (Cirrincione et al., 2018; Notararigo et al., 2013).

In fact, the EPS production and secretion are very unstable features and strongly dependent on diverse factors such as the microbial species, composition of the medium (carbon and nitrogen sources, growth factors, etc.), growth conditions, i.e. temperature, pH, oxygen tension, and incubation time (Degeest et

al., 2001; Loeffler et al., 2020). Moreover, nutrient, physical stresses and the effect of co-cultivation has been reported to affect the EPS production by LAB (Nguyen et al., 2020)

EPSs can exist in two forms, based on their locations: i) cell-bound EPSs, adhering to the cell surface (as capsular EPS), or ii) released EPSs into the growth medium (as free EPSs). Moreover, they can be classified as ropy or non-ropy (Mende et al., 2016). This phenotype is evident on agar plates where colonies of ropy strains produce long filament when extended with an inoculation loop; conversely, non-ropy strains do not produce any strands (Cirrincione et al., 2018). Furthermore, a particular attention should be given also to the determination of EPS chemical structure as well as the composition, as they both have an important role in the interaction with the food matrix and, as a consequence, they can differently modulate the rheology of the final product (Zhou et al., 2019).

Among LAB, *Lactobacillus delbrueckii* subsp. *bulgaricus* is recognized as an important starter in dairy industry (Xu et al.,2019). Among its peculiar characteristics, of particular importance is the ability to produce EPS *in situ.* Currently, *in situ* production of EPSs is of particular importance in the manufacture of a great variety of fermented dairy products such as classical yogurt, drinking yogurt, fresh cheeses, cultured cream, or milk-based desserts- (Zhu et al., 2019). Their contribution to the increment in viscosity, prevention of syneresis and improvement of sensory and nutritional characteristics especially in milk-derived products has been widely observed for several years (Doleyres et al., 2005). EPSs from *L. delbrueckii* subsp. *bulgaricus* are heteropolysaccharides (HePS), with a various structure consisting of repeating units of monomers such as glucose, galactose, rhamnose, and sometimes fructose (Sánchez-Medina et al., 2007,; Shene and Bravo, 2007; Zhou et al., 2019). Despite the EPS biosynthetic pathway is common to different bacterial species, intraspecific differences were also observed in the structure produced by *L. delbrueckii* subsp. *bulgaricus* species (Zhou et al., 2019).

Considering these differences, further studies are needed to implement the knowledge about the differences in kinetic production among strains of the same species. To date, different methods have

been used to evaluate the EPS production by different LAB species (Bancalari et al., 2019; Rühmann et al., 2015), from the simplest visual inspection of the colonies, to the more complex high-throughput screening methods (Poulsen et al., 2019), and to the alternative rapid screening by using impedometric analysis (Bancalari et al., 2019).

In the present study we describe a new approach aimed at investigating the differences in EPS synthesis within *L. delbrueckii* subsp. *bulgaricus* species. By combining impedometric analysis, fluorescent-microscope observation and flow cytometry analysis, the maximum EPS production and release into the medium were detected, with the final aim to find out the stage when EPS production is maximized. Furthermore, we found that the EPS production depends also on the number of EPS-producer cells that are present within the same population.

## 2. Materials and methods

### 2.1 Strains and growth conditions

Three wild *L. delbrueckii* subsp. *bulgaricus* (*Ldb* 2214, *Ldb* 2285 and *Ldb* 2000) EPS positive strains, belonging to the collection of the Food Microbiology Unit of the Department of Food and Drugs of the University of Parma, previously isolated from dairy matrices, were used for the analysis. Strains, maintained at −80°C as frozen stock cultures in MRS broth (Oxoid, Ltd., Basingstoke, United Kingdom) containing 20% (v/v) glycerol, were recovered in MRS by two overnight sub-culturing steps (2% v/v) at 42°C. Then, two additional sub-culturing steps (2% v/v) in MRS added with 40 g/L of sucrose instead of glucose (called MRS-sucrose, MRSS) were performed for each strain at 37°C before preliminary screening to evaluate EPS production and impedometric analysis (Bancalari et al., 2019; Pv and Singh, 2009).

### 2.2 Preliminary screening for EPS-producing strains

To confirm the ability of the strains to produce EPS, they were plated on MRSS agar medium. Ten µl of each strain were spotted on agar plates and, after incubation for 48 h at 37°C in anaerobic conditions (AnaeroGen, Oxoid, Milan, Italy), the EPS producers strains were recognized by evaluating their ability to produce filament when extended with an inoculation loop (Bounaix et al., 2009).

2.3 Impedance measurement for detection of EPS production

The three strains were analyzed by means of BacTrac 4300® Microbiological Analyzer system (Sylab, Austria). BacTrac 4300®, consisting of two incubators allowing simultaneous setting of four different temperatures. The equipment enables the separate registration of two specific impedance values, the *E*-value (E%) which is the relative change in capacitance at the electrode surface, called capacitance, and the *M*-Value (M%) which is the overall change in conductivity in the medium, called conductance (Bancalari et al., 2016). Both measures were recorded every 10 min for 40 h. The E% was considered for the evaluation of EPS production and growth behavior of the strains (Bancalari et al., 2019).

To evaluate, through impedometric analysis EPS production and growth behavior, the recovered strains were inoculated (2% v/v) into previously sterilized measuring BacTrac 4300® vials filled with 6 mL of MRSS, and incubated at 37°C. The analyses were carried out in triplicate and the collected E% data, at the end of analysis, were used to compare the impedometric curves and growth parameters (Bancalari et al., 2016) and to estimate ΔE as index of EPS production (Bancalari et al., 2019). The parameters obtained from the impedometric analysis were used to describe the growth kinetics of the strains. In particular, Lag value that is measured in hours, quantifies the time that the strains need to adapt before starting to grow. Thus, the greater the value, the bigger the time that the strains need to adapt to the growth conditions. Rate value describes the acidification speed and yEnd is the highest variation of impedance recorded, thus the maximum acidifying capacity of the strains (Bancalari et al.,2016)."

Four sampling points were settled, as reported in Fig. 1, and subjected to subsequent analysis, that were carried out in triplicate.

## 2.4 Microscopy analysis of EPS production

The EPS production by the three strains was followed during growth, by means of fluorescence microscopy through a Nikon Eclipse 80i epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a C-SHG1 100 W mercury lamp. Samples were stained with both Concanavalin-A (ConA), Alexa Fluor™488 Conjugate (Sigma-Aldrich, St Louis, USA) that binds selectively to mannopyranosyl and a-glucopyranosil of EPS, and 4',6-Diamidino-2- Phenylindole, di-hydrochloride (DAPI) (Sigma-Aldrich) to observe bacterial cells. Stocks solution of ConA (5 mg/ml) and DAPI (1 mg/ml) were prepared in 0.1M sodium bicarbonate (VWR, Milan, Italy) and milliQ water, respectively (Bancalari et al.,2019). ConA (7.5 µg/ml) and DAPI (1.5 µg/ml) were added to 1 mL of culture sample in a tube, mixed and incubated for 30 min at room temperature in the dark prior to observation.

## 2.5 Flow cytometry analysis

For the assessment of the total bacterial concentration, cell viability and EPS production, samples at different time points were diluted in PBS (pH 7.4) and analyzed by flow cytometry (FCM). For the assessment of viability, the cell suspensions were subjected to a simultaneous double staining with SYTO™ 24 1 µM (Thermo Fisher Scientific, Italy) and propidium iodide (PI) 2 µM (Sigma Aldrich, Italy), for 15 min, at 37 °C in the dark (ISO 19344, 2015). SYTO™ 24 permeates the membranes of all the cells in suspension and stains the nucleic acids with green fluorescence. PI penetrates only bacteria with damaged membranes, causing a reduction in SYTO™ 24 fluorescence when both dyes are present. Thus, live bacteria with intact cell membranes fluoresce bright green (indicated as active fluorescent cells),

bacteria with slightly damaged membranes exhibit both green and red fluorescence (indicated as damaged cells), whereas dead bacteria with broken membranes fluoresce red (defined as non-active fluorescent cells) (ISO 19344, 2015).

For the analysis of EPS production, samples were diluted in water and stained with Concanavalin A, Alexa Fluor™ 488 Conjugate (Thermo Fisher Scientific, Italy).

All the samples were analyzed using the flow cytometer with the following threshold settings: FSC 3,000; SSC 1,000; and 30,000 total events collected. All the parameters were collected as logarithmic signals. The rate of events in the flow was generally lower than 2,000 events/s. The obtained data were analyzed using BD Accuri™ C6 software version 1.0 (BD Biosciences, Milan, Italy). SYTO™ 24 (excitation 488 nm, emission 530/30 nm) and Alexa Fluor® (excitation 488 nm, emission 519 nm) fluorescence intensities of the stained cells were recovered in the FL1 channel. PI fluorescence (excitation 488 nm, emission filter 630/30 nm) intensity of the stained cells was recovered in the FL3 channel.

### 2.6 Statistical analysis

To investigate the differences observed among the strains, a two-way analysis of variance (ANOVA) model was performed using SPSS Statistics v. 25 (IBM, Armonk, NY, USA). One-way ANOVA and Tukey's HSD post hoc test were applied to test significant differences ($P < 0.05$).

## 3. Results

The visual inspection of the classical colony phenotype for EPS production after incubation on sucrose-supplemented agar medium (Bounaix et al., 2009), allowed to observe that each of the tested strains was able to produce slimy colonies after 48 h of incubation, confirming their capacity to produce EPS (Fig. 2). Then, strains were used for kinetic studies of EPS production.

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164 3.1 Strains growth kinetics

165 The strains growth kinetics were studied by means of two different methods: i) impedometric analysis for
166 the estimation of Lag phase length (Lag), acidifying velocity (Rate) and capacity (yEnd) (Bancalari et al.,
167 2016), and ii) Flow cytometry analysis (FCM) for the selective count of live, dead and damaged cells (Fig.
168 3). The impedometric curves are represented in Fig. 3 as grey line, as a mean of three replicates of E%
169 values. The shape of the grey curves obtained was different among the tested strains. In particular, the
170 curves of the two *L. delbrueckii* subsp. *bulgaricus* strains, namely *Ldb* 2214 and *Ldb* 2285 (Fig. 3a and 3b
171 respectively), were similar in shape and for the maximum E% value recorded. In fact, they reached the
172 maximum E% value around 10 hours of incubation. These results mean that these two strains showed a
173 similar acidification capacity, indicated by yEnd values of 28.2 for *Ldb* 2214 and 27.7 for *Ldb* 2285,
174 respectively, as shown in Table 1. As the bigger the yEnd value, the highest the acid production, this results
175 are in agreement with a previous study aimed at evaluating the acidifying abilities of starter lactic acid
176 bacteria (Bancalari et al., 2016). On the other hand, the rate value of *Ldb* 2214 was higher (5.1) than that
177 of *Ldb* 2285 (3.5), thus, showing a faster acidification rate. Regarding the lasting of the Lag phase, the two
178 strains showed values lower than 1h. Conversely, the E% curves of *Ldb* 2000 reveal its slowest growth as
179 compared to the other two strains (Fig. 3c). In particular, the calculated rate values were lower for these
180 strains (1.47), meaning a slowest growth rate. Furthermore, the highest Lag value (2.7h) was recorded for
181 this strain, confirming its slowest growth compared to the other two. In addition, also the acidifying
182 capacity was lower, with a calculated yEnd value of 14.9.
183 This intraspecific diversity in the acidifying characteristics has been already observed for this species in a
184 previous study (Bancalari et al., 2016), where the species *L. delbrueckii* subsp. *bulgaricus* were the most
185 heterogeneous among the 80 strains analyzed.

In parallel, the growth of selected strains was monitored by FCM and at each time point, cell suspensions were evaluated for the total cell number and for the selective quantification of live cells. FCM data confirmed the differences in growth kinetic observed by impedometric analysis. Despite the initial inoculum was similar (Table 1), the growth kinetics of the strains were different, mainly in terms of growth velocity. In particular, after 8 h of incubation and growth monitoring, strains *Ldb* 2214 and *Ldb* 2285 reached the maximum cell concentration (9.3 ± 0.1 log cells/ml and 9.1 ± 0.1 log cells/ml, respectively). Conversely, strain *Ldb* 2000 reached the maximum cell concentration after 23 h of incubation (8.9 ± 0.1 log cells/ml). As expected, around 95% of the cells were alive at the end of the incubation.

3.2 EPS production

The E% values extrapolated from the impedometric analysis were used to evaluate the strains EPS production, by using the parameter ΔE. This value was obtained by the differences between the maximum value of E% recorded and the E% value after 40 h of incubation to better highlight the differences. The obtained value describes the entity drop of the curve, which is an indication of EPS production (Bancalari et al., 2019).

ΔE values were variable among the strains, with the statistically highest value ($p<0.05$) of 9.16±0.12 recorded for *Ldb* 2214, then 8.21±0.20 for *Ldb* 2000 and 7.63±0.24 for *Ldb* 2285. This data showed that the acquisition of the capacitance values was more affected by the presence of the EPS produced by the strains *Ldb* 2214.

Observing the impedometric curves it was possible to note that the maximum production occurred after 8 h of incubation (T2), for the strains *Ldb* 2214 and *Ldb* 2285. Conversely, *Ldb* 2000 showed its maximum production at 15 h (Fig. 3).

EPS production was also evaluated by FCM. Cells were stained with Concanavalin A- Alexa Fluor™488 Conjugate, which binds selectively to mannopyranosyl and a-glucopyranosyl linkage of EPS. EPS production was evaluated at the inoculum, and then after 4, 8 and 23 h of incubation as measured fluorescence after the binding of the fluorophore to the specific residue of EPS. This probe has been already successfully used in the *in situ* detection of EPS both in cell cultures and in foods (Arltoft et al., 2007; Bancalari et al., 2020a), but, to our knowledge, this is the first time that this probe is applied for EPS detection by FCM.

The total emitted fluorescence measured (grey bars, Fig.3) has been directly related to the amount and composition of cell bounded-EPS. The results showed differences among the strains, and in particular, a pick of EPS production was found for the strains *Ldb* 2285 and *Ldb* 2214 after 8 h of incubation (namely, early stationary phase of growth, according to Bancalari et al, 2016). Conversely, for the strain *Ldb* 2000 the highest pick has been found already at T0, likely due to EPS already present on the surface of cells used to inoculate the fresh MRS. Despite the values recorded for each strain cannot be compared, as the composition of the produced EPS might be different from strain to strain, on the other hand, it could be very useful to describe the kinetics of EPS production and release into the environment. High values of emitted fluorescence correspond to a high concentration of cells with attached EPS or EPS with higher target of the fluorophore. In particular, the highest intensity of fluorescence was found for the strain *Ldb* 2285 after 8 hours of incubation (T2) (Fig. 3b, Fig. 4b). This observation is strengthened by the microscopic observation at T2, where almost all the cells are surrounded by the EPS (Fig.4b). Conversely, a decrease of total fluorescence was evident after 23 h of incubation (namely, late stationary phase of growth) for *Ldb* 2214 (Fig. 3a), likely due to a possible release of EPS in the surrounding. A different kinetic of release has been observed for the other two strains *Ldb* 2285 and *Ldb* 2000. By observing the total emitted fluorescence, no EPS seemed to be released from *Ldb* 2285 between 8 and 23 hours (Fig. 3b).

Different was the behavior of *Ldb* 2000, which showed a decrement of total fluorescence between 8 and 15 hours, suggesting the potential release of EPS in the environment (Fig. 3c). This hypothesis was also supported by the microscopic observation of the cells at 8 h of incubation (Fig. 4c). In fact, at this sampling point, a lowest number of cells with the EPS anchored to the cells surface has been observed by means of microscopy observation, suggesting a greater release into the environment (Fig. 4c). Moreover, this highest EPS release could have interfered with the current flow between the electrodes, resulting in a flatter E% curve observed for the strain *Ldb* 2000 (Fig. 3c) (Bancalari et al., 2019).

It has been already reported that *L. delbrueckii* subsp. *bulgaricus* is able to produce HePS with a different structure depending on the strain and the growth conditions (Nishimura, 2014; Zhou et al., 2019). More specifically, it has been observed that several factors, mainly the biosynthesis position and the diverse interaction between enzyme and substrates in the Wzx/Wzy pathway, lead to structural diversity in HePS composition, resulting in an enormous difference in structure, from molecular composition to EPS structure and branching (Whitfield et al., 2020; Zhou et al., 2019). In fact, the complexity of Wzx/Wzy-dependent pathway, that has been found in lactobacilli, involves a high number of enzyme and interacting sites and it is the cause of the larger variability observed in the HePS structure (Zeidan et al., 2017). Moreover, as the probe used in our study strongly binds specific hetero-EPS (HePS) sites (Arltoft et al., 2007), we can hypothesize that for *Ldb* 2214 and *Ldb* 2000 a similar amount of total emitted fluorescence could be attributed to a more similar EPS composition.

Comparing the results of the impedometric analysis and FCM analysis, it could be noted that after 8 hours of incubation (T2), where the impedometric curves began to drop, also the maximum values of fluorescence were recorded for all *Ldb* strains, confirming the hypothesis that the maximum amount of EPS synthesis can be found at this stage of growth phase. Moreover, according to both approaches, different EPS production kinetics and release were detectable for the strains.

### 3.3 Phenotypic heterogeneity in EPS production

Beside the total fluorescence for the evaluation of EPS production, it is worth to underline that FCM is capable of analyzing billions of single cells simultaneously, providing measurements of fluorescence and physical parameters at single cell level, unravelling the heterogeneity of a cell population (McKinnon, 2018). In this context, after cell suspension staining with Concanavalin A- Alexa Fluor™488 Conjugate, FCM analysis revealed the heterogeneity of the single cultures in terms of EPS production. Indeed, only part of the cells displayed detectable fluorescence (Table 1, Fig. 5), indicating that only a fraction of the growing cells was able to produce EPS, recognized by the selected fluorophore. Although only part of the cell population was identified as EPS producer, an evident increase of positive events with EPS was observed between 4 and 8 hours of growth, in particular for strains *Ldb* 2214 and *Ldb* 2000. In specific, positive events for EPS were around 73% and 20.9% for *Ldb* 2214 and *Ldb* 2000, respectively. Conversely, EPS producer cell percentage was quite stable in *Ldb* 2285 (Table 1 and Fig. 5).

More in detail, after 8 h of incubation, when the maximum production is supposed to occur, *Ldb* 2000 and *Ldb* 2285, showed a concentration of EPS producer cells lower than *Ldb* 2214. In particular, 7.7 Log cell/ml and 7.3 Log cell/ml for *Ldb* 2085 and *Ldb* 2000, respectively (Table 1). Conversely, 9 Log cell/ml were positive for EPS fluorescence in *Ldb* 2014. This is of great interest as, in broad terms, 12.7 and 4.5% of the total population of *Ldb* 2000 and *Ldb* 2285 respectively have EPS attached to the cell surface. On the contrary, after 4 hours of incubation, *Ldb* 2214 showed a highest proportion of EPS-positive cells (67.2%, corresponding to 9.0 Log cell/ml).

After 8 hours of incubation, about 73% (9.2 Log cell/ml) of *Ldb* 2214 cells were identified as EPS producer, but only 10% (8.1 Log cell/ml) and 21% (7.6 Log cell/ml) were countable in *Ldb* 2285 and *Ldb* 2000, respectively.

At the end of the EPS production monitoring (23 h of incubation), 50% (9.1 Log cell/ml), 15.4% (8.4 Log cell/ml), and 11% (8.0 Log cell/ml) of the populations were stained as EPS producer in *Ldb* 2214, *Ldb*

2285, and *Ldb* 2000, respectively. In particular, data on *Ldb* 2214 confirmed the simultaneous decrease of the total fluorescence measured for this strain. Interestingly, this strain had also the highest percentage of cells with EPS (ranging from 12.0 and 53.1%, Fig. 5), but the lowest fluorescence. This effect could be due to the release of EPS into the medium.

The differences observed by comparing all the obtained results confirmed that the EPS production could be extremely variable also within the same species.

*Lactobacillus delbrueckii* subsp. *bulgaricus* was chosen for this study as it is one of the most commonly used species as starter cultures in dairy products for its multiple positive fermentation abilities (Bancalari et al., 2020b; Dan et al., 2019). In fact, besides its acidifying capacity, of great technological interest, is its potentiality to modulate the rheological characteristics of the fermented products through EPS production (Nishimura, 2014), and the flavor through the production of aroma compounds (Dan et al., 2019). Comparing the growth behaviors and EPS production kinetics of the three tested strains, interesting differences have come to light, underling that the EPS production kinetic is a fundamental information to be considered in the selection criteria for the strain to be used.

Even though different methods are available to select strains basing on their ability to produce EPS, our results indicate that this information may not be enough, and more detailed study about the kinetic of production and release are needed, in order to maximize the effect of EPS production and subsequent interaction in a food matrix.

## 4. Conclusions

In our study, the EPS production kinetics of 3 strains belonging to *L. delbrueckii* subsp. *bulgaricus* species, one of the most important starters employed in dairy sector, was evaluated. As the ability to produce EPS is one of the discriminating phenotypic traits in the selection of the strains to be used in dairy transformations, of major importance is to deep the knowledge about EPS synthesis before strain

selection. To better characterize the growth and EPS production kinetics, we combined several approaches: impedometric analysis, FCM and fluorescence microscopy.

Although the impedometric analysis allows to discriminate whether a strain is able to produce EPS, by using FCM we moved a step forward. In fact, by a selective staining of cells with a probe binding a specific EPS site, we were able to establish the kinetic of EPS production and release.

In particular, the used approach allowed to highlight the intraspecific diversity among the three strains in EPS production and release kinetics, in relation to the different growth phases. More specifically, we were able to identify that the maximum EPS production occurred between 4 and 8 hours, and then, depending on the strain, a possible release of EPS in the environment occurred.

The highest fluorescence values were measured for *Ldb* 2285, conversely, the lowest were detected for *Ldb* 2000 and *Ldb* 2214, who probably released the EPS in the medium.

Interestingly, the FCM allowed us to determine that only a part of the cell population of the strains was identified as EPS producer. This last finding needs further investigations to figure out the mechanism behind the observed heterogeneity in EPS production in a population with a homogeneous genetic background.

Our findings may contribute to a more specific selection of the strains depending on the intended use.

**Author contribution**

**Elena Bancalari:** Conceptualization; Data curation; Formal analysis; Investigation; Resources; Supervision; Visualization; Writing - original draft, Writing - review & editing; **Monica Gatti:** Conceptualization; Data curation, Resources; Supervision; Visualization; Writing - original draft, Writing - review & editing; **Benedetta Bottari:** Writing - review & editing; Resources, Data curation; **Diego Mora:** Writing - review & editing; Resources, Data curation; **Stefania Arioli:** Conceptualization; Data curation;

Formal analysis; Investigation; Resources; Supervision; Visualization; Writing - original draft, Writing - review & editing

## Declaration of competing interest

The authors declare that there are no conflicts of interest.

## Acknowledge

This research was partially supported by the SIMTREA (Italian Society of Agrifood and Environmental Microbiology): Dr Elena Bancalari was awarded the national mobility fellowship.

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Table1. Impedometric (Lag, rate, yEnd, ΔE%) and flow cytometry (EPS production, total cells count, live cells count and total emitted fluorescence) measurement of three *L. delbrueckii subsp. bulgaricus* strains (2214, 2285 and 2000) evaluated at four different sampling points.

| Species | Strain | Origin | Sampling points | Time (h) | *EPS production (events/µL) | *Total cells count (events/ml) | *Live cells count (events/ml) | Total emitted fluorescence (Fluorescent Unit, FU) | % of EPS producing cells | Lag values (hours) | Rate values | yEnd values | ΔE% |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *L. delbrueckii* subsp. *bulgaricus* | 2214 | Milk | T0 | 0 | 1.3E+07$^{b}$ | 1.1E+08$^{b}$ | 9.8E+07$^{b}$ | 9095 ± 455$^{b}$ | 12.0% ± 0.6 | <1±0.12$^{a}$ | 5.1±0.15$^{c}$ | 28.2±0.10$^{b}$ | 9.16±0.12$^{c}$ |
| | | | T1 | 4 | 1.0E+09$^{c}$ | 1.5E+09$^{c}$ | 1.4E+09$^{c}$ | 16814 ± 835$^{c}$ | 67.2% ± 3.4 | | | | |
| | | | T2 | 8 | 1.5E+09$^{d}$ | 2.0E+09$^{d}$ | 2.0E+09$^{d}$ | 23688 ± 1085$^{d}$ | 73.7% ± 3.7 | | | | |
| | | | T3 | 23 | 1.2E+09$^{e}$ | 2.4E+09$^{e}$ | 2.3E+09$^{e}$ | 11344 ± 567$^{e}$ | 50.1%± 2.5 | | | | |
| *L. delbrueckii* subsp. *bulgaricus* | 2285 | Milk | T0 | 0 | 1.1E+07$^{b}$ | 7.8E+07$^{b}$ | 6.7E+07$^{b}$ | 49156 ± 2350$^{b}$ | 14.3% ± 0.7 | <1±0.10$^{a}$ | 3.5±0.10$^{b}$ | 27.7±0.23$^{b}$ | 7.63±0.24$^{a}$ |
| | | | T1 | 4 | 4.6E+07$^{c}$ | 1.0E+09$^{c}$ | 9.8E+08$^{c}$ | 48083 ± 2389$^{b}$ | 4.5% ± 0.2 | | | | |
| | | | T2 | 8 | 1.4E+08$^{d}$ | 1.4E+09$^{d}$ | 1.3E+09$^{d}$ | 116407 ± 5632$^{c}$ | 10.0% ± 0.5 | | | | |
| | | | T3 | 23 | 2.2E+08$^{e}$ | 1.5E+09$^{d}$ | 1.4E+09$^{d}$ | 102276 ± 5085$^{d}$ | 15.4% ± 0.8 | | | | |
| *L. delbrueckii* subsp. *bulgaricus* | 2000 | Curd | T0 | 0 | 6.6E+05$^{b}$ | 3.8E+07$^{b}$ | 3.2E+07$^{b}$ | 26205 ± 1310$^{b}$ | 2.5% ± 0.1 | 2.7±0.09$^{b}$ | 1.47±0.21$^{a}$ | 14.9±0.10$^{a}$ | 8.21±0.20$^{b}$ |
| | | | T1 | 7 | 2.1E+07$^{c}$ | 1.7E+08$^{c}$ | 1.5E+08$^{c}$ | 24528 ± 1226$^{b}$ | 12.7% ± 0.6 | | | | |
| | | | T2 | 15 | 4.0E+07$^{d}$ | 2.0E+08$^{d}$ | 1.7E+08$^{d}$ | 16407 ± 820$^{c}$ | 20.2% ± 1.0 | | | | |
| | | | T3 | 23 | 4.7E+08$^{e}$ | 1.0E+09$^{e}$ | 8.8E+08$^{e}$ | 24693 ± 1234$^{b}$ | 46.7% ± 2.3 | | | | |

*Standard deviation less than 5%.

$^{a\text{-}e}$ lower case superscript letters (within a column of each strain) highlight differences and/or analogies among different time points for the same strain.

**Figure captions**

**Figure 1.** Schematic representation of the study

**Figure 2.** Example of the slimy colonies formed by the strains on MRSS agar medium

**Figure 3.** Growth curves (red and blue lines, corresponding to viable and total cell counting, respectively), EPS production kinetics (grey histograms) and quantification of EPS-producer cells (green line) of *L. delbrueckii* subsp. *bulgaricus* strains. A) *Ldb* 2214; b) *Ldb* 2285; c) *Ldb* 2000. E% values (grey line) is also indicated.

**Figure 4.** Microscopic observation of the strains: a) *Ldb* 2214; b) *Ldb* 2285 and c) *Ldb*2000 at T2.

**Figure 5**. Dot plot of FCM analysis to evaluate EPS production heterogeneity. FSC-H: forward scatter of cell suspension; FL1-H: green fluorescence of Alexa Fluor. The green gate encompasses EPS-producer cells; outside this gate, unstained cells (non-producer cells or cells which had already released EPS into the medium. The percentage represent the mean of 3 independent assays. Standard error is less than 5%.