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Abstract

Bacterial production of exopolysaccharides (EPS) is of increasing interest near food manufacturers, biotechnology industries and nutritionists because of their different roles. Several analytical methods are available for recovery, quantification and characterization of EPS from lactic acid bacteria (LAB) in food. However, direct screening method for production of EPS is still based on the visual observation of filamentous texture of the colonies developed on supplemented solid growth media. To overcome weaknesses of many currently used screening methods, we propose adopting impedance microbiology to evaluate the EPS production from LAB in milk. In this work we have proven that the peculiar shape of capacitance curve of *Lactobacillus delbrueckii* subsp. *bulgaricus* 2214, measured in milk by means of a BacTrac 4300® system, is due to production of EPS. Besides the pH measurement, the amounts of EPS evaluated after 0, 8, 13 and 55 hours of incubation in milk, were in agreement with the evaluation of gene expression and confirmed by the observations by confocal laser scanning microscopy and by transmission electron microscopy.

With the aim to verify the applicability of the proposed method, the drop entity of the capacitance curve (ΔE%) of 22 EPS-producing (EPS+) LAB strains and one negative (EPS-) control was evaluated both in broth medium and in milk. The positive ΔE% value found for all of the strains cultivated in the clear broth medium allowed to confirm the EPS production, simply observing a strain-dependent amount of EPS on surface of the measurement electrodes of the device. When the same EPS+ strains were cultivated in milk, the obtained ΔE% values showed that only a few of them were able to produce EPS in this environment, supporting their diversified ability to utilize lactose for this purpose.

Results obtained by this multidisciplinary study demonstrate that impedance microbiology represents a suitable method to overcome the limits of the most commonly used methods to screen LAB for EPS production in milk. Moreover, these results also open a door to the application to other food and beverages, in which the EPS produced *in situ* could be of great interest for food industry.

Keywords: milk, conductance, confocal laser microscopy, transmission electron microscopy

1. Introduction

Bacterial exopolysaccharides (EPS) have been largely described as they are important metabolites, with variable composition and physiochemical characteristics. This structural variability offers a large set of physico-chemical and biological properties (Badel et al., 2001; Ruas-Madiedo and De Los Reyes-Gavilán, 2005). Some authors describe EPS as loosely associated to cells, some others as released into extracellular medium, or sometimes are indicated with the term solely to describe those polysaccharides that are not covalently bound to the bacteria and only exist free in the environment (Lynch et al., 2018). There are many types of EPS that can be classified based on whether the main-chain polymer consists of a single monosaccharide-type (i.e. homopolysaccharides) or more than one (i.e. heteropolysaccharides) (Badel et al., 2011).

Different roles are attributed to bacterial EPS, such as structure stabilizers in biofilm, signaling molecules in cellular recognition, and quorum-sensing control mediators. However, their physiological functions are still relatively unknown and only few of them have been exploited in industrial applications (Badel et al., 2011). Furthermore, EPS might represent an extracellular energy/carbon reserve. However, this role is questionable since most of EPS-producing (EPS+) species lack the genes involved in the EPS degradation (Torino et al., 2015; Zannini et al., 2015).

EPS produced by lactic acid bacteria (LAB), because of their safe character, attract a lot of interest by food industries, mostly because regulating their use in food products would be much simple (Badel et al., 2011; Zannini et al., 2015). In this respect, LAB can be regarded as microbial factories for the production of safe metabolites of technological interest (Boguta et al., 2015; Torino et al., 2015). LAB EPSs could, totally or partly, replace plant- or seaweed-derived hydrocolloids, or milk solids, that are currently used in food manufacturing to improve viscosity, texture, stability and mouthfeel of final products. Particularly, LAB EPS may improve the rheological and sensory

characteristics of fermented dairy products such as yogurt and fresh cheeses, avoiding unwanted whey separation during fermentation or upon storage (Galle and Arendt, 2014; Lynch et al., 2018).

Recently, the increasing demand for natural polymers for industrial application in the food sector has led to a renewed interest in EPS from LAB, especially for the *in situ* production (Zannini et al., 2015). The EPS production by LAB, from genes to applications in food, has been thoroughly reviewed by Zeidan and colleagues (Zeidan et al., 2017). Moreover, the commonly used methods for recovery, quantification and characterization of LAB EPS in food have been recently reviewed (Leroy and De Vuyst, 2016; Lynch et al., 2018).

Despite this growing interest, and the great variety of different techniques described to identify polysaccharides-producing microorganisms, screening methods have received less attention. Two main classes of screening strategies can be found, based on either the screening for EPS-producing strains or for phenotypes associated with the presence of these polysaccharides (Poulsen et al., 2019). The visual inspection of slimy or mucoid colonies on solid media, changes in viscosity or texture analysis are commonly used to identify if the EPS production occurs.

Alternatively, the use of degenerate primers could be a rapid method to evaluate the presence of a target gene involved in the EPS production pathway (Lynch et al., 2018). However, the positive result of this evaluation is not always associated with the expected phenotype.

Taking into account the limits of the above-mentioned screening approaches, we propose to apply the impedance microbiology to directly and easily detect EPS-producing-LAB in a complex food matrix such as milk.

When applied to microbiology, impedance can be defined as the resistance in flow of an alternating electrical current that passes through a conducting microbial culture medium where microorganisms develop during the incubation period (Lanzanova et al., 1993; Mucchetti et al., 1994). Thus, by measuring the electric signal that quantifies the movement of ions between two electrodes, impedance microbiology can be used to evaluate LAB growth in a culture medium (Yang and Bashir, 2008). The continuous plot of the recorded data results in a conductance or capacitance

curves (Bancalari et al., 2016). The instrument used for the analysis, e.g. BacTrac 4300®, allows the measurement and plotting of two impedance components: (i) the variation of impedance of culture medium (Zm), which is recorded as the relative change in conductivity compared to an initially recorded value, and visualized as M% change (conductance) over time; (ii) the variation of the electrode system (Ze), caused by the ionic double layer in the vicinity of the surface of the two electrodes (Futschik et al.,1995), which is also recorded and visualized as E% changes (capacitance) over time.

Differently from the conventional applications of this technique, we would like to propose impedance measurement as an innovative approach to screen EPS-producing LAB.

With the aim to provide sufficient scientific support to our hypothesis, the measurement of impedance changes, pH value, level of gene expression, observations by confocal laser scanning microscopy and by transmission electron microscopy have been initially applied to study the feasibility of the proposed approach on one EPS-producing LAB strain (Fig.1). The reliability of the method was successively confirmed on a set of other LAB strains evaluated for their capability to produce EPS in broth culture and milk.

2. Materials and Methods

2.1 Relative measure of capacitance for *Lactobacillus delbrueckii* subsp. *bulgaricus* 2214

Lactobacillus delbrueckii subsp. *bulgaricus* 2214 *(Ldb* 2214*)*, maintained at −80°C as frozen stock cultures in MRS (Oxoid, Ltd., Basingstoke, United Kingdom), was recovered in MRS broth by two overnight sub-culturing steps (2% v/v) at 42°C. Two additional overnight sub-culturing steps (2% v/v) were performed at 37°C in skim milk powder (Oxoid Ltd.), previously reconstituted to 10% (w/v) and sterilized at 100°C for 15 min (SSM). A 2% (v/v) inoculum from the last sub-culturing step was added to 18ml of either MRS broth or SSM. This volume was then equally divided into three sterilized measuring vials and analyzed in triplicate at 37°C by means of a BacTrac 4300® Microbiological Analyzer system (Sylab, Austria). This instrument consists of two incubators

allowing simultaneous setting of four different temperatures and was used to evaluate the impedometric curves. The values of the two specific impedance parameters, M% and E%, were both recorded every 10 min during 55-h incubation and, for the present study, the E% was considered as the most sensitive measure.

Four samples were collected at different sampling times, i.e. just after *inoculum* (T0) and after 8 h (T1), 13 h (T2) and 55 h (T3) of incubation. One negative sample, consisting of non-inoculated SSM, was also incubated as negative control.

The pH value was measured by means of a pHmeter Beckman Instrument mod Φ350 (Furlenton, CA, USA) with a Hamilton glass electrode (Bonaduz, Switzerland). The sampled aliquots were used for further analyses. The experiments were carried out in triplicate. Non-inoculated SSM was also incubated as negative control.

2.2 Expression of the genes involved in the EPS production by *Lactobacillus delbrueckii* subsp. *bulgaricus* 2214 in SSM

At each sampling time point (T0, T1, T2 and T3), total genomic DNA was extracted using the DNeasy Kit (Qiagen, Milan, Italy) following the manufacturer's instructions. The DNA concentration was determined using a Cary 50 Spectrophotometer (Varian Inc., Torino, Italy) and checked by agarose gel electrophoresis. The genomic DNA was diluted to 20 ng/µL for PCR.

Cell pellets obtained after centrifugation (10 min at 10,000 rpm) were grinded with liquid nitrogen using mortar and pestle. The RNA was extracted using the RNeasy Kit (Qiagen) following the manufacturer's instructions. RNA concentration and purity were determined using a Cary 50 Spectrophotometer, checked by agarose gel electrophoresis, and reverse transcribed into cDNA with Quantiscript Reverse Transcriptase (QuantiTect Reverse Transcription Kit, Qiagen) using random hexamer primers according to the manufacturer's instructions.

The *epsE* gene, a phospho-glucosyltransferase from the *gtf* region of *Lb. delbrueckii* subsp. *bulgaricus* (accession number AAG44709.1) (Lamothe et al., 2002), was used for homologous

sequences identification through BLASTx in NCBI database. The BLASTx alignment evidenced a genetic similarity of 98% with four *Lb. delbrueckii* sequences related with sugar transferase gene (WP_014565346.1, WP_035176038.1, WP_011678627.1, WP_011544274.1). These sequences were aligned for primers designer using DNAMAN software (version 4.15, Lynnon Biosoft Company, San Ramon, CA, USA). The forward primer Ld *epsE* for CTGAGAAGCTGAAGAAGGATCTG and the reverse primer Ld *epsE* rev AGTGACATATTCCCAATCAGCAC were identified in the coding region, which yielded an amplification product of 140bp. The partial fragments of the *epsE* gene of *Ldb* 2214 strain were amplified using the primers Ld *epsE* for-Ld *epsE* rev on genomic DNA. PCR reactions were composed of 7 µL of sterile MilliQ water, 10 µl of 2-PCR GoTaq Master Mix (Promega, Madison, WI, USA), 1 µl of forward primer (10 mM), 1 µl of reverse primer (10 mM) and 1 µl of template DNA (20 ng/µl). The following thermal cycling conditions were used: initial strand denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final extension step at 72 °C for 7 min. The resulting amplicons were purified using the QIA quick PCR purification Kit (Qiagen), sequenced by MACROGEN Europe (Amsterdam, The Netherlands) and aligned using DNAMAN software.

Relative quantification was performed using the QuantStudio 3 (Applied Biosystems, Carlsbad, CA, USA) with the Power SYBR Green PCR Master Mix (Applied Biosystems). The 20 µl PCR reaction included 1 µl of cDNA, 0.5 µl of forward primer, 0.5 µl of reverse primer and 10 µl of SYBR green. The reactions were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The fluorescence signal was acquired at 60°C. Melting curve analysis (60-95°C with a heating rate of 0.1°C per second and a continuous fluorescence measurement) was carried out. After the reaction, the Ct data were determined using default threshold settings, and the mean Ct was determined from the triplicate PCRs. The specific gene primers Ld *epsE* for-Ld *epsE* rev were used for the relative quantification using 16S rRNA as a reference gene (Table 1). 16S rRNA primers were designed for the amplification of the *Lb. delbrueckii* subsp. *bulgaricus* species.

The fold changes at T1, T2 and T3, were obtained with respect to the T0 using the DDCT method (Livak and Schmittgen, 2001). The real-time PCR amplification efficiencies (E) in the exponential phase were calculated according to the equation: E=10(-1/slope). The obtained E values were 91% and 92%, for *epsE* and 16S rRNA respectively, with a difference lower than 5% between the two genes.

2.3 Quantification of exopolysaccharides in SSM

The procedure for isolation of free EPSs previously reported by Mende (Mende et al., 2012) was used with slight modifications. Five mL of sample were added with 0.7 mL 80% (w/v) trichloroacetic acid (TCA) and heated at 90°C for 15 min in a water bath. Samples were cooled in ice water, centrifuged (2000 rpm, 20 min, 4°C) to remove cells and protein, and the supernatant was neutralized with NaOH. The EPSs were purified following the procedure reported by Rimada and Abraham (Rimada and Abraham, 2003). Samples were centrifuged (20 min at 2600 rpm) and the pellets were dried at 55°C. The dried material was assumed as the EPS amount, which was thus expressed as polymer dry mass (PDM) (Van Geel-Schutten et al., 1999, Degeest et al., 2001, Lin and Chien 2007).

2.4 Confocal laser scanning microscopy and transmission electron microscopy of EPS produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* 2214 in SSM

EPS produced by *Ldb* 2214 were inspected in cultures taken at each sampling point (T0, T1, T2 and T3) from duplicate experiments, using confocal laser scanning microscopy (CLSM) and, for sample T2 only, transmission electron microscopy (TEM). Specimens for CLSM were prepared by staining with either Concanavalin-A (ConA), Alexa Fluor™ 488 Conjugate (Sigma-Aldrich, St Louis, USA) that binds selectively to mannopyranosyl and a-glucopyranosyl of EPS, or 4',6-Diamidino-2-Phenylindole, di-hydrochloride (DAPI) (Sigma-Aldrich) to observe bacterial cells. The staining was performed as follows: stocks solution of ConA (5 mg/ml) and DAPI (1 mg/ml) were prepared in 0.1

M sodium bicarbonate (VWR, Milan, Italy) and milliQ water, respectively. The culture samples (250 µl) were added with 50 µl of ConA and 15 µl of DAPI in an eppendorf tube, mixed and incubated for 30 min at room temperature prior to CLSM observation. An inverted CLSM equipment A1 from Nikon (Minato, Japan) was used. ConA was excited at the wavelength of 488 nm using an argon laser and the emission filter was set at 515-545 nm. DAPI was excited at the wavelength of 405 nm and the emission filter was set at 420-460 nm. 3D-images for image analysis consisted of 512 x 512 pixels stack images that were captured with separation between layers set at 0.30 µm. Image analysis was performed using ImageJ software (Research Services Branch, National Institute of Health and Medicine, USA) on maximum projection of CLSM z-stack images. Nuclei counting plugin was used to count the number of bacteria in the CLSM images under the conditions described by D'Incecco and colleagues (D'Incecco et al., 2018a). Numbers of counted bacteria are the mean of three independent measurements.

Specimens for TEM were prepared using the conventional negative stain protocol. Briefly, a small drop of sample was adsorbed onto a carbon-coated copper grid, washed with two drops of deionized water, and stained with two drops of freshly prepared 1% water solution of uranyl acetate (EMS, Hatfield, USA). Samples were imaged using a Philips E208 TEM (Aachen, Germany) operating at an acceleration voltage of 80 kV as described by D'Incecco and colleagues (D'Incecco et al., 2018b).

Twenty microscopic fields were observed and representative images have been selected.

2.5 Preliminary screening for EPS producing strains

One hundred LAB strains able to produce EPS were preliminarily identified by the visual inspection of the colonies on solid growth media MRS or M17 supplemented with sucrose. Screened strains were from LAB species commonly known as EPS producer (*Lb. delbrueckii* subsp. *bulgaricus, Lactobacillus casei, Lactobacillus helveticus, Lactobacillus paracasei, Lactobacillus plantarum, and Leuconostoc spp., Streptococcus thermophilus, and Weissella spp.)* and belonged to the

Laboratory of Food Microbiology of the Department of Food and Drugs of the University of Parma or to the ATCC or LMG collections. Strains were maintained as frozen stock cultures at −80°C in MRS or M17broth (Oxoid, Ltd., Basingstoke, United Kingdom) containing 20% (v/v) glycerol. *Lactobacillus* and *Weissella* strains were all recovered in MRS, whereas *Streptococcus* and *Leuconostoc* in M17 broth. Two overnight sub-culturing steps (2% v/v) were carried out at 42°C for *Lb. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, and at 30°C for the strains belonging to the other species. Then, two additional sub-culturing steps (2% v/v) in either MRS or M17, both added with 40 g/L of sucrose (Oxoid, Ltd., Basingstoke, United Kingdom) instead of glucose (called MRS-sucrose, MRSS, and M17-sucrose, M17S, respectively), were performed for each strain at 37 or 30°C, depending on the species. The recovered strains were plated on agar plates and incubated at 37°C or 30°C for 48 hours. Strains able to produce slimy or mucoid colonies (Bounaix et al., 2009) were selected for the study.

2.6 Impedance measurement for detection of EPS production

Twenty-two LAB strains, able to produce EPSs according to the preliminary phenotype investigation, and one negative control *S. thermophilus* 530 (Table 2), were analyzed by mean of impedance measurement. The 23 strains, recovered by means of two sub-culturing steps, were inoculated (2% v/v) in 18ml of MRSS or M17S broth or in SSM. This volume was then equally divided in to 3 previously sterilized measuring vials and analyzed in triplicate at 37°C or 30°C by mean of BacTrac 4300®.

With this instrument, bacteria are detected in real time via the decrease of the impedance in an alternating current (AC) field. Any impedance change caused by the bacterial metabolism is detected. The BacTrac 4300® is based on the impedance splitting method and is able to register two specific impedance values for each single measurement: i) the conventional M-value (media impedance) and ii) E-value which is the electrochemical double layer of the electrodes-electrolyte impedance. Both these value are shown as relative changes compared to a starting value and

252 expressed as M% and E%. This opens the possibility to evaluate the results based on two different
253 signals. For registration of the electrode impedance value the capacitive component of the complex
254 impedance value Z (influence of the electrochemical double layer of the electrode) is of central
255 importance. The technique to measure this electrode impedance value is a special characteristic of
256 the BacTrac4300® measurement technology. The E-value is not significantly influenced by the
257 composition of the growth media and is therefore fundamental when media with high salt content
258 are used and this significantly broadens the possible applications
259 (https://microbiology.sylab.com/products/p/show/Product/product/bactrac-4300.htm).
260 This instrument requires the use of glass measuring cells with 4 electrodes inside. Once the vials are
261 aseptically filled with the samples, these are located into the appropriate position inside the BacTrac
262 4300® incubators that can be used independently whenever samples are available to be tested. The
263 measuring system is accurate, stabile and flexible, and can handle even media with a high salt
264 content and the scope of applications is not limited to any matrix
265 (https://microbiology.sylab.com/products/p/show/Product/product/bactrac-4300.html). E%
266 parameter was recorded every 10 minutes for 55hours by the Bac-Win software (Sy-Lab) to a
267 central database, which allows the real-time visualization of the impedance curves. Data collected at
268 the end of incubation period were exported from the dedicate program Bac-Eval software (Sy-Lab)
269 plus and used to calculate the parameter ΔE% that was assumed as an indicator of EPS production.
270 The measurements were carried out in triplicate. Negative control samples were also incubated,
271 consisting of non-inoculated MRSS, M17S and SSM.
272

273 **3. Results**

274 **3.1 Capacitance curve and EPS production of *Lactobacillus delbrueckii subsp. bulgaricus 2214***
275 Relative measure of capacitance of EPS+ *Ldb* 2214 was evaluated at 37°C, instead of optimal
276 growth temperature of 42°C, in order to stimulate EPS production, as previously demonstrated for

Lactobacillus spp. (Kim et al., 2008, Polak-Berecka et al., 2014). Capacitance curve of EPS+ *Ldb* 2214, obtained by the continuous plotting of the modification of E value (E%) during the incubation at 37°C up to 55 hours, is shown in Figure 2. Usually, the capacitance curve is similar to a typical bacterial growth curve. After a short initial phase, comparable to the lag phase, it rapidly develops in a phase comparable to an exponential growth phase. However, differently from the bacterial growth curve, capacitance curve mainly depends on metabolism of the inoculated cells, not only on their number (Bancalari et al., 2016). In the case of *Lbd* 2214, instead, a progressive decrease in the capacitance value (E%) was observed after 13 hours of incubation (T2). As capacitance is not a measure of cell death, and the drop of the curve cannot be comparable to the death phase of the bacterial growth curve, we hypothesized that the curve drop was due to EPS production. To prove this hypothesis, four sampling times were chosen that best characterized the curve shape of *Lbd* 2214 grown in SSM (Fig. 2). For each sampling, besides the pH measurement, the following aspects were considered and evaluated: i) level of gene expression involved in the EPS production, ii) amount of EPS produced (as PDM), and iii) EPS characterization by both CLSM and TEM.

As expected, due to the typical acidifying activity of the LAB metabolisms, the pH value decreased from 6.3 to 4.5 within the first 8 h of incubation (T1), then slowly reached the value 3.4 after 55 h (T3) (Table 3). The *epsE* gene expression (relative quantification, Rq) was below the detection threshold in T0 and reached the maximum value of 1.0±0.035 after 8 h (T1) (Table 3). Since the capacitance curve partially reflects the bacterial growth curve, T1 corresponds to the middle of the exponential growth phase (Bancalari et al., 2016). In the subsequent sampling points, the *epsE* gene expression gradually decreased. The amount of EPS increased progressively to the maximum value of 4.64±0.06 g/L in T2 then decreased to 2.87±0.05 g/L at 55 h (Table 3).

3.2 Confocal laser scanning microscopy and transmission electron microscopy of *Lactobacillus delbrueckii* subsp. *bulgaricus* 2214 grown in SSM

Few single cells of *Lbd* 2214 were observed by CLSM at T0 (Fig. 3a), whereas mainly cells aggregates were present at the subsequent sampling points (Fig. 3). The number of bacteria cells, counted by image analysis on three different images per sample, progressively increased from 184 cells at T0 to 932 cells at T2, and then decreased to 535 at T3 (Fig. 3). EPSs emitting bright green fluorescence were detected at all points, except for T0. Both volume and fluorescence of EPS increased up to a maximum at T2, then decreased at T3 in accordance with the EPS quantification (Table 3). As expected, any fluorescence was detected in non-inoculated milk (not shown).

Interestingly, the combination of fluorescence probes here adopted for staining either EPS or *Ldb* 2214 cells allowed us to highlight that they exactly overlapped (Fig. 4) and thus that bacterial cells were trapped within the EPS matrix. Small cell-free EPS particles were also observed (Fig. 4b), consistently with the presence of soluble EPS reported by Nielsen and Jahn (1999). Soluble EPSs are poorly studied so far, being the available information mostly related to the bound EPS (Sheng et al., 2010).

Sample T2 was analysed also by TEM (Fig. 5). The observation at ultrastructural level confirmed that bacterial cells were surrounded by EPS. Numerous casein micelles were trapped within the EPS network creating an electron-dense coarse structure (Fig. 5a, 5b). Two different types of EPS were clearly distinguishible: one was stuck to the bacterial cell surface in a smooth layer (Fig, 5c), the other was rather present as filaments elongating from the bacterial cell and likely used to bind to the protein matrix (Fig. 5b).

3.3 Application of the proposed method

With the aim to verify the applicability of impedance measurement to detect EPS production, the capacitance curves of 22 EPS+ and one EPS- LAB strains, were performed in triplicate in MRSS or M17S and in SSM.

As previously reported, the shape of the capacitance curve has to be considered, since a drop of the curve was an evidence of EPSs production. This phenomenon can be objectively described through

328 the parameter ΔE%, calculated as the difference between the maximum value reached by E% and
329 the value recorded after 55 hours of incubation. The ΔE% values were calculated from triplicate
330 experiments (Table 2). Values were always positive for the EPS+ strains developed in MRSS or
331 M17S broth, varying considerably among the strains (Table 2). *Ldb* 2214 and *S. thermophilus* 111
332 showed the highest ΔE% values, whereas *Lc. citreum* 4454 and 4461 as well as *Lb. casei* 334 the
333 lowest ones (Table 2). Given the clearness of broth medium, a gelatinous biofilm was easily
334 observable around the electrodes in the measurement vials for all of the EPS+ strains, thus
335 confirming the effectiveness of the proposed approach (Fig. 6).
336 When the same strains were grown in SSM, the ΔE% values were lower than in broths, although to
337 a different extent, and intriguingly were negative for 78% of the strains (Table 2). Due to milk
338 turbidity, the possible presence of EPS was visually detectable only by opening the vial at the end
339 of incubation to inspect the electrodes (Fig. 7). In the vials of the five strains with a ΔE% higher
340 than 3, we observed the presence of filamentous and sticky substance covering the electrodes (data
341 not shown). In contrast, the electrodes in the vials of all the other strains were clean and similar to
342 those of EPS- *S. thermophilus* 530. This observation suggested their inability to produce EPS in
343 milk (data not shown).
344
345 **4. Discussion**
346 The impedance measurement is based on a principle that dates back to 1899 (Stewart, 1899) but its
347 application in food microbiology is quite recent and mainly associated with the rapid detection of
348 foodborne pathogenic bacteria (Yang and Bashir, 2008). This technique enables qualitative and
349 quantitative tracing of microorganism growth by measuring the change in the electrical conductivity
350 of the culture medium where they develop. This technique is generally used for a rapid detection of
351 foodborne pathogenic bacteria and the most common way to use this measurement is by fixing a
352 point, generally defined as "time of detection" that coincides with the reaching of a cell
353 concentration of about 10^6-10^7 CFU/ml (Bancalari et al., 2016). Otherwise, it was recently used to

evaluate the starter LAB acidifying performances with a different use of impedance data recorded (Bancalari et al., 2016). This approach, that provides three parameters by fitting the data obtained by Gomperz equation, had the limit that the original and fitted curves obtained have to overlap (Fig. 8). This means that the curves must have a shape like that of a microbial growth curve. This was not the case of *Ldb* 2214, whose capacitance curve recorded during growth in milk, after some hours of incubation, started to decrease (Fig. 8). As capacitance is not a measure of cells death, we hypothesized that the production of EPS and its accumulation on the electrodes could be the cause of the descending trend of the E% curve. With the aim to provide scientific support to prove that the positive value of ΔE% was due to the EPS production in milk, it was decided to evaluate EPS production at different growing steps of *Ldb* 2214 by following different approaches. Indirectly, through the study of the *epsE* gene expression, and directly, by quantifying the EPS as dry weight of the ethanol-insoluble fraction (Goh et al., 2005) and attaining their ultrastructural characterization using both CLSM and TEM.

In LAB, genes encoding for EPS-biosynthesis proteins are typically organized in clusters with an operon structure and, in the eps operon, genes can be categorized into groups based on the putative or established functions of their products (Zeidan et al., 2017). The biosynthesis of EPS involves the build-up of individual repeating units on a lipid carrier by the sequential activity of glycosyltransferases (GTFs) that, therefore, are key enzymes for the biosynthesis process. The GTFs catalyze the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, thereby forming a glycosidic bond (Goudenège, et al., 2014; Van Kranenburg et al., 1999; Zeidan et al., 2017). In LAB, GTFs are located in the central region of the eps clusters (Zivkovic et al., 2015).

The *epsE* gene was chosen as the target gene for this study, as it is expected to encode a priming GTF that would initiate the biosynthesis of the repeating unit by linking a phosphate-sugar to the lipophilic carrier (Lamothe et al., 2002). With the aim of establishing a link between gene expression and phenotype, the amount of produced EPS was quantified as PDM. The PDM method

380 is based on the extraction of the total polymers that are insoluble in ethanol. Therefore, the presence
381 of small amounts of low molecular weight carbohydrates could bring to slight EPS overestimation
382 (Goh et al., 2005). This could explain why, in our experiments, a small amount (< 1 g/L) of PDM
383 was quantified also immediately after the *inoculum* (Table 3). This fraction was not detected by
384 CLSM analysis (Fig. 3a). The gene expression was below the detection threshold in T0, reached its
385 maximum value in T1 with a fold-change equal to 1, when cells were expected to be in their
386 exponential growth phase, and then gradually decreased in T2 (0.16 fold-change) and T3 (0.005
387 fold-change). These data showed that the *epsE* gene is activated within the first 8 hours, in
388 agreement with the observed production of EPS, which increased sharply in the first 8 h and more
389 slowly in the subsequent step, i.e. from T1 to T2 (Table 3). The further increment in this second
390 step, despite the decrease of *epsE* gene expression, was probably due to the fact that EPS synthesis
391 mainly occurs during the exponential growth, when the gene expression was greater, resulting in an
392 accumulation of EPS at T2 (Table 3). Consistently with this observation, higher numbers of bacteria
393 cells and greater production of EPS were concomitantly observed by the CSLM analysis at T1 and
394 T2 (Fig. 3b, 3c).
395 Interestingly, both the total EPS quantity and the relative gene expression decreased at T3. Previous
396 studies have shown the EPS content to decline during a prolonged fermentation. A decrease in EPS
397 amount may be the result of: i) a physiologically changing cell environment (Gancel and Novel,
398 1994a; Gancel and Novel, 1994b), ii) the degradation by glycol-hydrolytic activity (Cerning et al.,
399 1992; Pham et al., 2000), or iii) reversible DNA rearrangements resulting in different cell types with
400 different exo-polymer production capacities (De Vuyst and Degeest, 1999). Furthermore, the same
401 authors also stated that both temperature and pH could influence EPS degradation during
402 fermentation (De Vuyst and Degeest, 1999).
403 The EPS produced by *Ldb* 2214 proved to have a very complex ultrastructure. We observed the
404 presence of both EPS trapped within the protein matrix or the capsular EPS layer, tightly bound to
405 the cell. The latter is also known as "glycocalyx", described as polysaccharides layered on the

406 bacteria surface together with glycoproteins (Ruas-Madiedo and De Los Reyes-Gavilán, 2005). In
407 accordance with our results, the production of both capsular and ropy EPS was previously observed
408 for *Lb. delbrueckii* spp. *bulgaricus* (Hassan et al., 1995). About the EPS composition, we can
409 speculate that we were dealing with hetero-EPS, since the lectin ConA has high affinity for binding
410 to them (Arltoft et al., 2007). Our TEM observations suggested that EPS formed a network-like
411 structure capable of entrapping protein or even small casein micelles. Also supported by the
412 evidences obtained by Ayala-Hernandez and colleagues (Ayala-Hernandez et al., 2008) using the
413 scanning electron microscopy, we can state that EPS interacted with the protein matrix and were not
414 simply located in void spaces. These tight interactions might explain the presence of some intact
415 casein micelles in SSM at pH as low as 3.9 (Table 3) due to the lactic acid fermentation, the main
416 energy metabolism of *Ldb* 2214. Ayala-Hernandez et al., (2008) (also demonstrated that EPS
417 molecules might interact not only with casein but also with whey proteins, playing an active role in
418 the formation of aggregates and thus improving the viscosity of milk fermented by EPS-producing
419 LAB.
420 To sum up briefly, the explanation of our approach is based on two points: i) the meaning of
421 "capacitance", that is the double layer capacitance of the electrodes/electrolyte interface impedance;
422 ii) the capacitance modification (recorded as percentage variation, E%) during LAB growth is
423 strongly affected by ionic layers in the vicinity of the electrode surface (Futschik et al., 1995).
424 The value of the double layer capacitance is extremely sensitive to slight alteration of the surface of
425 the electrodes-electrode impedance, and this is the reason why it was decided to use it. In fact, this
426 sensitive measure allowed us to detect the EPS-produced, whenever they were attached to the
427 surface of the electrodes or suspended in the nearby.
428 When the EPS-producing LAB grows in milk, inside the measure cells where the electrodes are
429 located, they modify both conductance and capacitance mainly because of the conversion of the
430 uncharged lactose (○ in Fig. 9) into the smaller and charged lactic acid (● in Fig. 9) during the
431 incubation time (from t0 to t3 Fig. 9). Resulting conductance or capacitance curves are similar and

comparable to a typical bacterial growth curve (Fig. 9). Nevertheless, in case of EPS production (◊ in Fig.9) and regardless whether they are charged or not, EPS stick on the electrode surface, due to their own adhesive properties and/or their affinity for the electrodes, changing the composition of the compact layers (Futschik et al., 1995). Adhesion of EPS to the electrode surface slightly alters the interface impedance by blocking the registration of electrical impedance at the area of contact, thus causing the descent of the capacitance curves (Fig. 9).

Considering that the evidences obtained from our study on EPS + *Ldb* 2214 concordantly support the initial hypothesis that the production of EPS causes a positive ΔE% value, we have applied this method to 22 strains EPS+ in broth (MRSS and M17S). A strain-dependent amount of EPS at the surface of the measurement electrodes of the BacTrac 4300® (Fig. 6) was easily observed in the transparent media and it was always combined with a positive value of ΔE%.

Possibly, the extent of ΔE% decrease and also the beginning of curve drop could be affected by a different chemical composition or a different amount of EPS. All these aspects are not elucidated yet, and need to be deeply investigated in further researches.

For the moment, this method can be considered as an efficient screening approach. In the screening step we have carried out, this method allowed us to easily observe that only few strains showed a ΔE% value >3 when cultivated in milk. According with Bauer and colleagues (Bauer et al., 2009), who observed that only a limited number of microorganisms produced EPS using lactose, the only sugar present in SSM,-we can state that, although considered EPS+, the LAB strains with a negative value of ΔE% were not able to produce EPS in SSM. On the contrary, the strains with positive ΔE% were able to utilize lactose to produce EPS in SSM.

In conclusion, the impedometric approach used in this study to detect the production of EPS in milk could overcome the limits of the most common used screening methods. The entire measuring cycle is automatic, therefore it is less laborious, and it is not highly demanding in terms of technical skills compared to the methods based on colonies observation on solid growth media. It does not need

large volume of samples, as rheometer and texture analysis do, and differently from genetic methods, it allows to evaluate the LAB phenotype.

Since production of EPS is a very unstable feature, although this method does not give direct information about the nature or the amount of the EPS produced, it could be easily applicable for the study of the best conditions for *in situ* production that are linked to different bacterial growth parameters. Moreover, since a high number of variable can be tasted at the same time (pH, temperature, sugar concentration) and gets results in very few hours, this approach could find application at least in two different industrial realities. The dairy starter cultures manufacturers could apply it for rapid screening of the strains. The dairy industries could apply it to define the best technological parameters as to have the *in situ* EPS production with the aim to develop or improve new products. Moreover, beyond milk, these results leave an open door to the application to other food and beverages, in which the EPS produced *in situ* could be of great interest.

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Figure Legends

Fig. 1. Scheme of the approach used to demonstrate our hypothesis and confirm the applicability of the proposed method.

Fig. 2. Capacitance curve of *Lb. delbrueckii subsp bulgaricus* 2214, inoculated in SSM and incubated at 37°C for 60 h. Solid line corresponds to the average curve obtained from three independent experiments and dashed lines indicate SD interval. T0-T3: sampling times.

Fig. 3. CLSM of *Lactobacillus delbrueckii* subps. *bulgaricus* 2214 inoculated in SSM and incubated at 37°C. Images correspond to the four sampling time points: (a) T0 is the sample collected to the moment of inoculum. (b) T1, (c) T2 and (d) T3 samples were collected after 8, 13 and 55 hours of incubation at 37°C, respectively. Bacteria cells labelled with DAPI appear in blue and EPS labelled with ConA appear in green. Enlarged detail of bacteria cells within the EPS matrix at T2 is shown in panel c'. Scale bars are 10 μm in length.

Fig. 4. CLSM of *Lactobacillus delbrueckii* subps. *bulgaricus* 2214 and EPS in SSM, after 13 h of incubation at 37°C (T2). Split channels showed (a) bacteria cells labelled with DAPI in blue and (b) EPS labelled with ConA in bright green. Small fractions of cell-free soluble EPS (S-EPS) were found free to move in the sample. Scale bars are 10 μm in length.

Fig. 5. TEM micrographs of EPS produced by *Lactobacillus delbrueckii* subps. *bulgaricus* 2214 in SSM. Negative stain with uranyl acetate evidenced bacterial cells (B), casein micelles (C) trapped within the EPS (arrows) network where also pores (P) are visible. Panels “b” and “c” are enlarged areas of the white frames in panel “a”. Loosely bound EPS (LB-EPS) interact with casein micelles while the tightly bound EPS (TB-EPS) form a capsule around the bacterium. Scale bar is 500 nm in length in panel “a” and 250 nm in length in panels “b” and “c”.

Fig. 6. Visual observation of mucoid EPS produced in MRSS by *Lactobacillus delbrueckii* subsp. *bulgaricus* 2214. a) front view of the vials at the end of incubation in the BacTrac 4300®; b) top view of EPS on the surface of the electrodes.

Fig. 7. Visual observation of mucoid EPS produced in SSM by *Lactobacillus delbrueckii* subsp. *bulgaricus* 2214. a) front view of vial opened at the end of incubation in the BacTrac 4300®, b) clot observed from top of the vial, c) EPS on the surface of the electrodes after clot discharge.

Fig. 8. Capacitance curves of (a) *Streptococcus thermophilus* 530 EPS- strain and (b) *Lactobacillus delbrueckii subsp. bulgaricus* 2214 EPS+ strain, fitted to the Modified Gompertz equation (Gibson et al., 1988) using DMfit version 2.1 Excel add-in (http://www.combase.cc/index.php/en/tools). Blue diamond symbols are the y values that DMfit uses to represent the E% data recorded by the BacTrac4300 each 10 minutes for 60 hours of incubation. Red solid line (Fit 1) is the fitted curve described by Modified Gompertz equation.
For the EPS- strain (a), the fitted curve is represented by a sigmoidal curve that well overlaps the original one. In this case, three parameters can be calculated by the ComBase tool which are useful to describe and interpret the curve: i) lag time (λ), ii) maximum specific E% rate (μmax), and iii) maximum value of E% (Yend) (Bancalari et al., 2016). The possibility to fit the original data to the Modified Gompertz equation is tied to the necessity that the two curves overlap (Bancalari et al., 2016). In the case of EPS+ strain (b), the two curves do not overlap. Thus, to describe the curve with only one parameter, the drop entity of the capacitance signal was arbitrarily calculated by the parameter ΔE%, i.e. the difference between the maximum recorded value of E% and the E% value at 55h of incubation.

Fig. 9. Schematic outline of the conversion mechanisms causing change of the capacitance curve (electrode impedance) E% at the four descriptive moments of LAB EPS- and LAB EPS+ strains growth in milk. Magnification of the electrode surface and of the molecules mainly involved in electrochemical phase at boundary metal/electrolytes: lactose (○), lactic acid (●) and EPS (◊).

676 **Table 1**. Primers and PCR conditions used to detect genes related to EPS synthesis in *Lb.*

677 *delbrueckii* subsp. *bulgaricus* 2214 strain.

Gene	Primer sequence	Annealing temperature	Amp.size (bp)	Reference
Ld *epsE* for Ld *epsE* rev	CTGAGAAGCTGAAGAAGGATCTG AGTGACATATTCCCAATCAGCAC	58	140	This study
TBA-FW TBA-RV	CGGCAACGAGCGCAACCC CCATTGTAGCACGTGTGTAGCC	63	130	Denman and McSweeney, 2006

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680 **Table 2**. Mean values ± standard deviation of ΔE% for the strains cultivated in MRSS or M17S and

681 SSM. All the analyses were carried out in triplicate

Species	**strains**	**ΔE%**	
		MRSS or M17S	**SSM**
Lactobacillus casei	ATCC 334	4.90±0.36	-10.31±0.47
Lactobacillus casei	4339	17.42±0.14	0.83±0.31
Lactobacillus delbrueckii subsp. *bulgaricus*	1932	15.24±0.37	13.15±0.50
Lactobacillus delbrueckii subsp. *bulgaricus*	2214	24.12±0.41	14.05±1.42
Lactobacillus delbrueckii subsp. *bulgaricus*	1870	10.88±0.41	0.05±0.15
Lactobacillus helveticus	Lh 23	14.53±0.45	4.87±0.48
Lactobacillus helveticus	Lh 28	14.25±0.49	4.74±0.27
Lactobacillus paracasei	4340	18.91±0.37	0.01±0.22
Lactobacillus paracasei	4341	19.28±0.37	-13.28±0.42
Lactobacillus paracasei	4366	22.83±0.35	-10.93±0.14
Lactobacillus plantarum	LMG 18399	22.24±0.20	-6.86±0.30
Leuconostoc	4454	7.07±0.47	-26.78±0.49
Leuconostoc	4461	8.01±0.24	-24.14±0.46
Streptococcus thermophilus	530	-0.04±0.05	-0.04±0.05
Streptococcus thermophilus	111	23.65±0.47	-0.35±0.44
Streptococcus thermophilus	113	9.55±0.46	-0.24±0.17
Streptococcus thermophilus	114	15.47±0.49	-0.24±0.23
Streptococcus thermophilus	145	20.70±0.5	3.78±0.50
Streptococcus thermophilus	159	12.58±0.54	-0.04±0.35
Streptococcus thermophilus	161	11.84±0.46	-0.27±0.26
Streptococcus thermophilus	192	18.92±0.50	-0.37±0.12

Weissella	4451	16.53±0.45	1.75±0.31
Weissella	4458	18.07±0.46	1.51±0.24

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684 **Table 3.** Mean value ± standard deviation of pH, Relative quantification (Rq) of the *epsE* gene
685 expression, and total EPS amount as polymer dry mass (PDM) (g/L) at each sampling time of
686 *Lactobacillus delbrueckii* subsp. *bulgaricus* 2214, inoculated in SSM and incubated at 37 °C for 55
687 h. Values are the mean of three measurements

688

Sampling time	**pH**	**Rq**	**PDM (g/L)**
T0= *inoculum*	6.3±0.0	0.005±0.002	0.88±0.01
T1= 8h	4.5±0.0	1.0±0.035	3.60±0.05
T2= 13h	3.9±0.0	0.16±0.036	4.64±0.06
T3= 55h	3.4±0.0	0.005±0.002	2.87±0.05

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Figure 1

FIG 1. Scheme of the approach used to demonstrate our hypothesis and confirm the applicability of the proposed method.

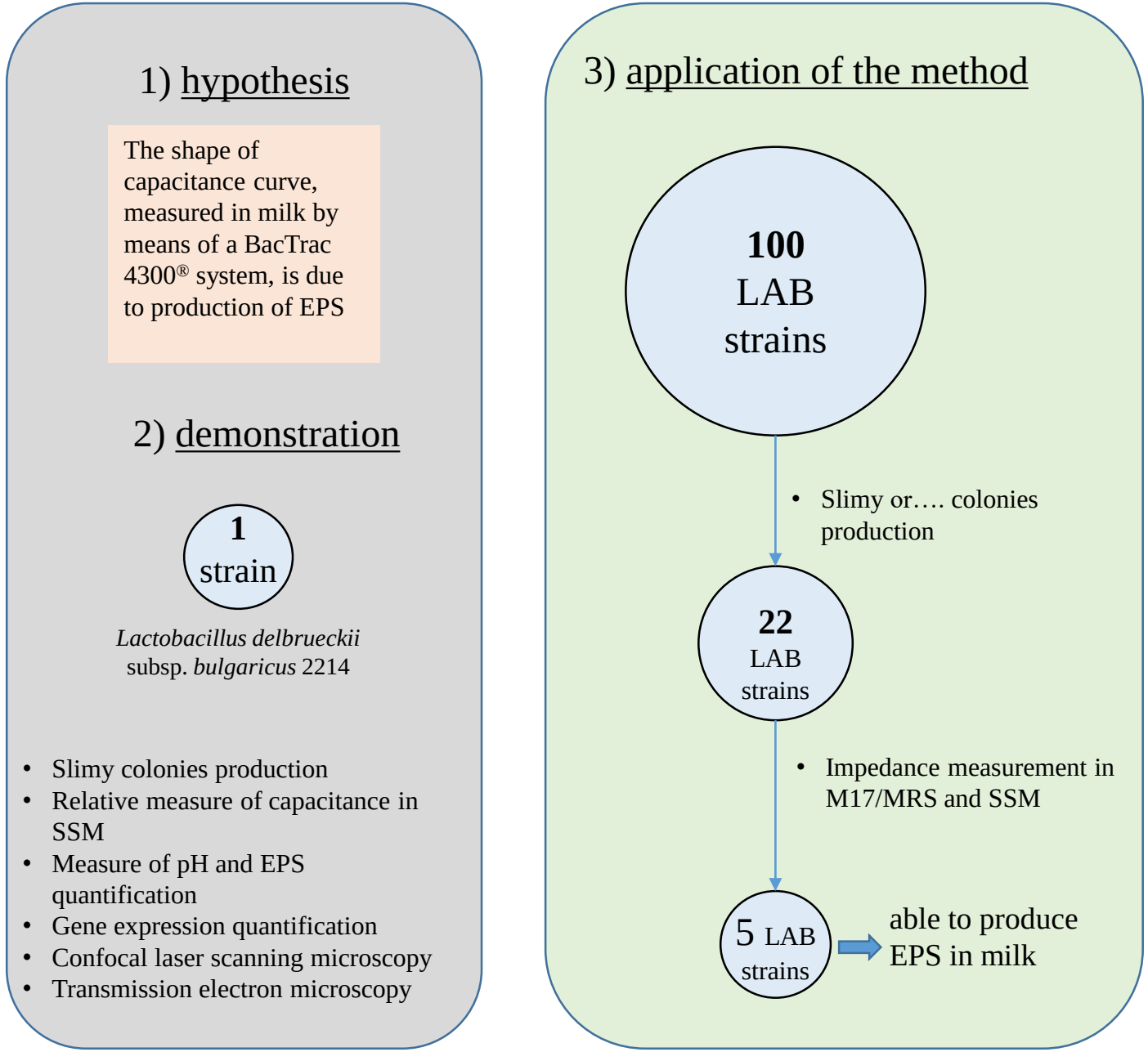
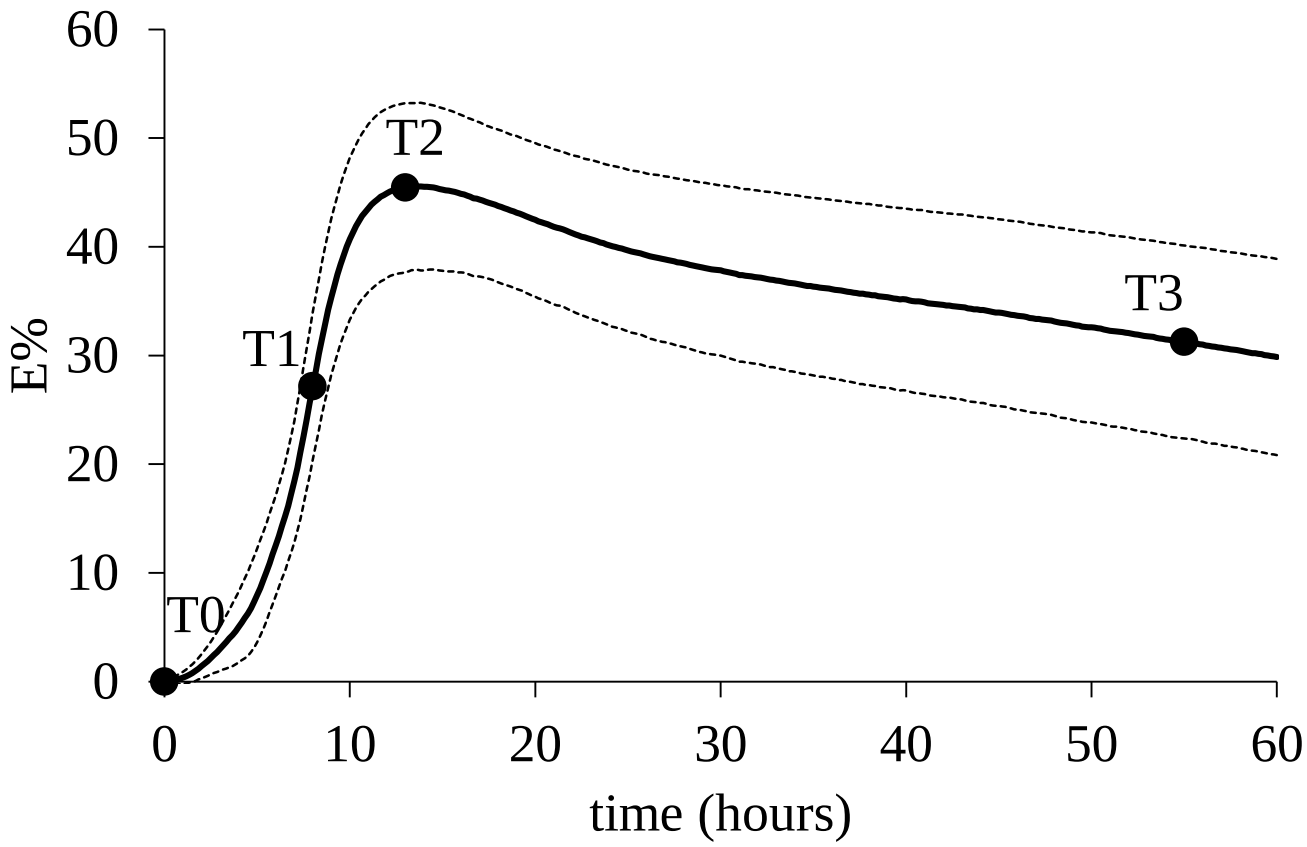


Figure 2

FIG 2 Capacitance curve of *Lb. delbrueckii* subsp *bulgaricus* 2214, inoculated in SSM and incubated at 37°C for 60 h. Solid line corresponds to the average curve obtained from three independent experiments and dashed lines indicate SD interval. T0-T3: sampling times. .



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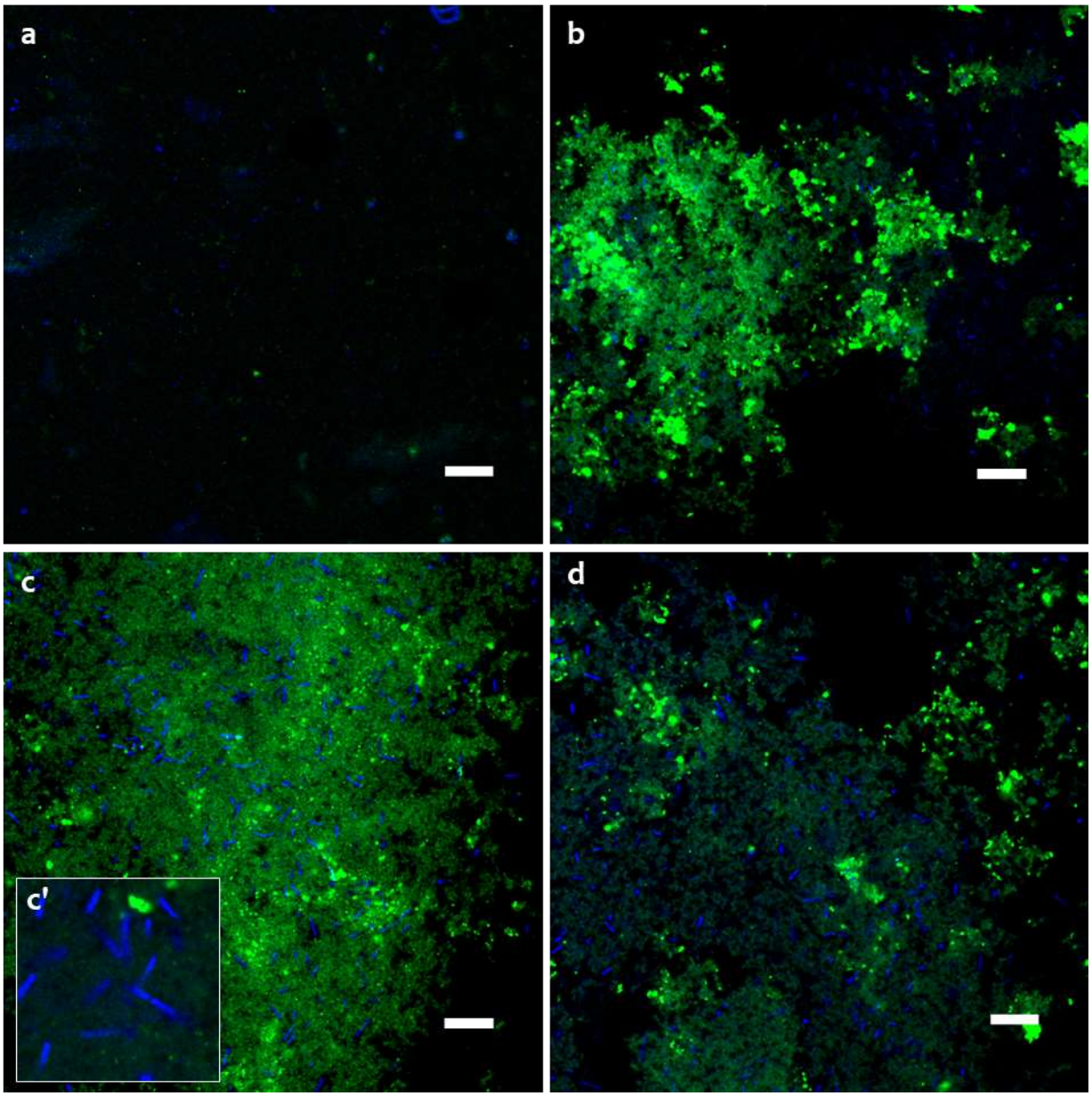

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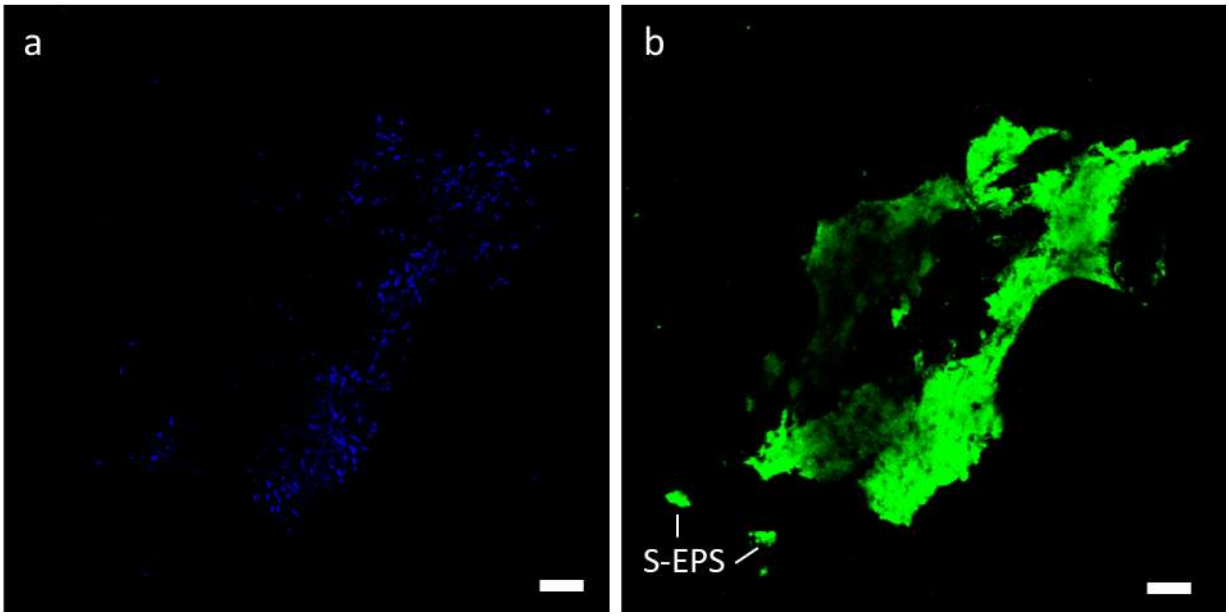

Figure 5

FIG 5 TEM micrographs of EPS produced by *Lactobacillus delbrueckii* subps. *bulgaricus* 2214 in SSM. Negative stain with uranyl acetate evidenced bacterial cells (B), casein micelles (C) trapped within the EPS (arrows) network where also pores (P) are visible. Panels "b" and "c" are enlarged areas of the white frames in panel "a". Loosely bound EPS (LB-EPS) interact with casein micelles while the tightly bound EPS (TB-EPS) form a capsule around the bacterium. Scale bar is 500 nm in length in panel "a" and 250 nm in length in panels "b" and "c".

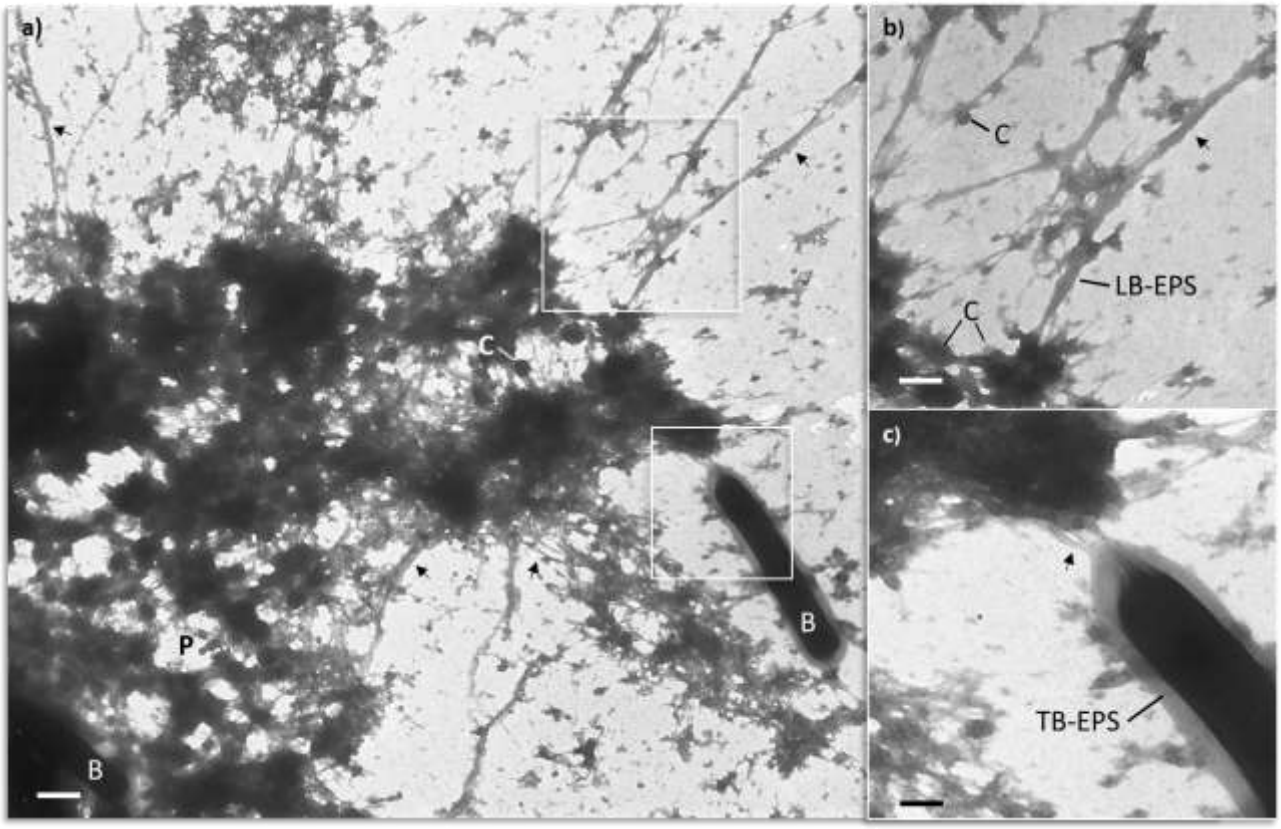

Figure 6

FIG 6 Visual observation of mucoid EPS produced in MRSS by *Lactobacillus delbrueckii* subsp. *bulgaricus* 2214. a) front view of the vials at the end of incubation in the BacTrac 4300®; b) top view of EPS on the surface of the electrodes.

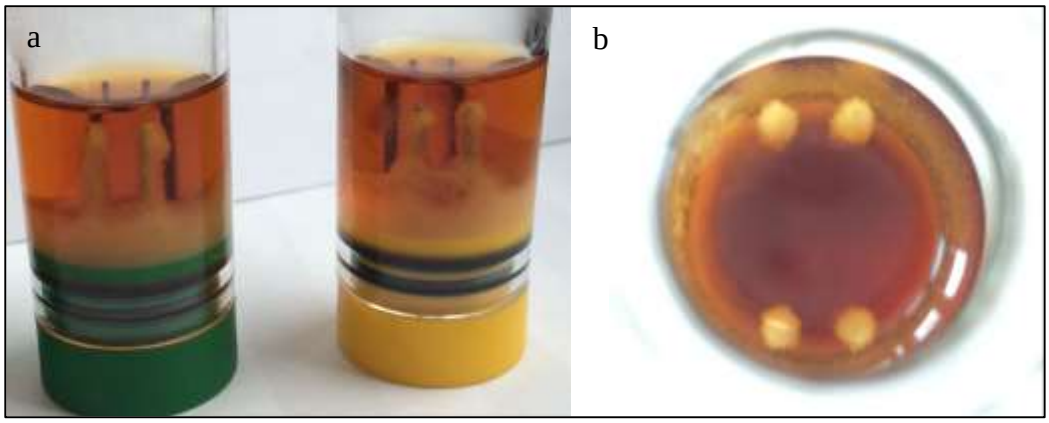

Figure 7

FIG 7 Visual observation of mucoid EPS produced in SSM by *Lactobacillus delbrueckii* subsp. *bulgaricus* 2214. a) front view of vial opened at the end of incubation in the BacTrac 4300®, b) clot observed from top of the vial, c) EPS on the surface of the electrodes after clot discharge.

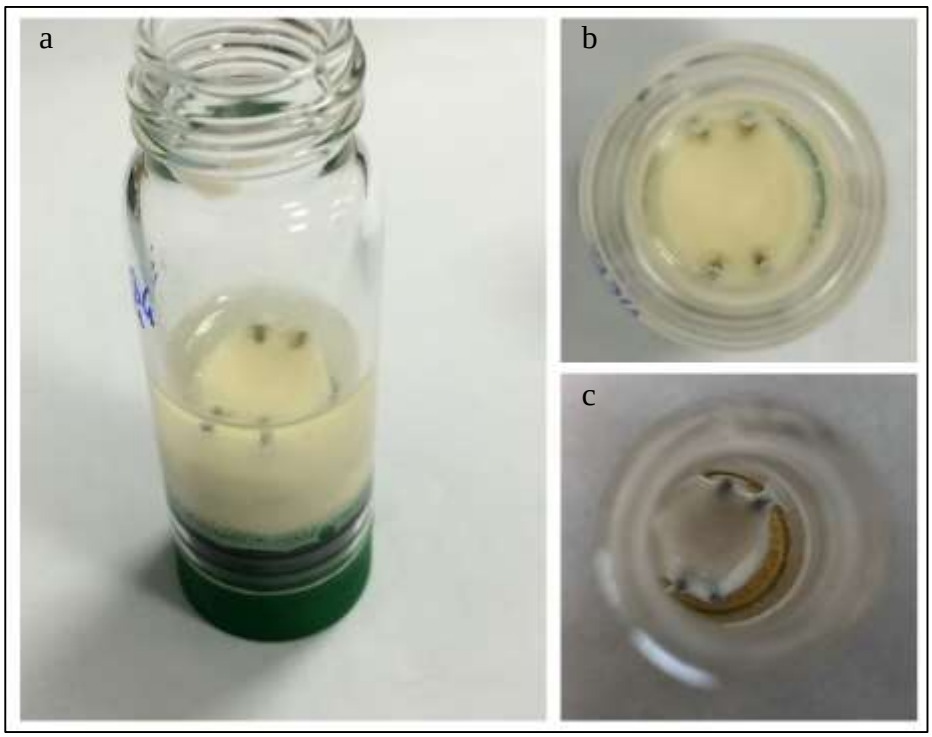

Figure 8

FIG 8. Capacitance curves of (a) *Streptococcus thermophilus* 530 EPS- strain and (b) *Lactobacillus delbrueckii* subsp. *bulgaricus* 2214 EPS+ strain, fitted to the Modified Gompertz equation (Gibson et al., 1988) using DMfit version 2.1 Excel add-in (http://www.combase.cc/index.php/en/tools). Blue diamond symbols are the y values that DMfit uses to represent the E% data recorded by the BacTrac4300 each 10 minutes for 60 hours of incubation. Red solid line (Fit 1) is the fitted curve described by Modified Gompertz equation.
For the EPS- strain (a), the fitted curve is represented by a sigmoidal curve that well overlaps the original one. In this case, three parameters can be calculated by the ComBase tool which are useful to describe and interpret the curve: i) lag time (λ), ii) maximum specific E% rate (μmax), and iii) maximum value of E% (Yend) (Bancalari et al., 2016). The possibility to fit the original data to the Modified Gompertz equation is tied to the necessity that the two curves overlap (Bancalari et al., 2016). In the case of EPS+ strain (b), the two curves do not overlap. Thus, to describe the curve with only one parameter, the drop entity of the capacitance signal was arbitrarily calculated by the parameter ΔE%, i.e. the difference between the maximum recorded value of E% and the E% value at 55h of incubation.

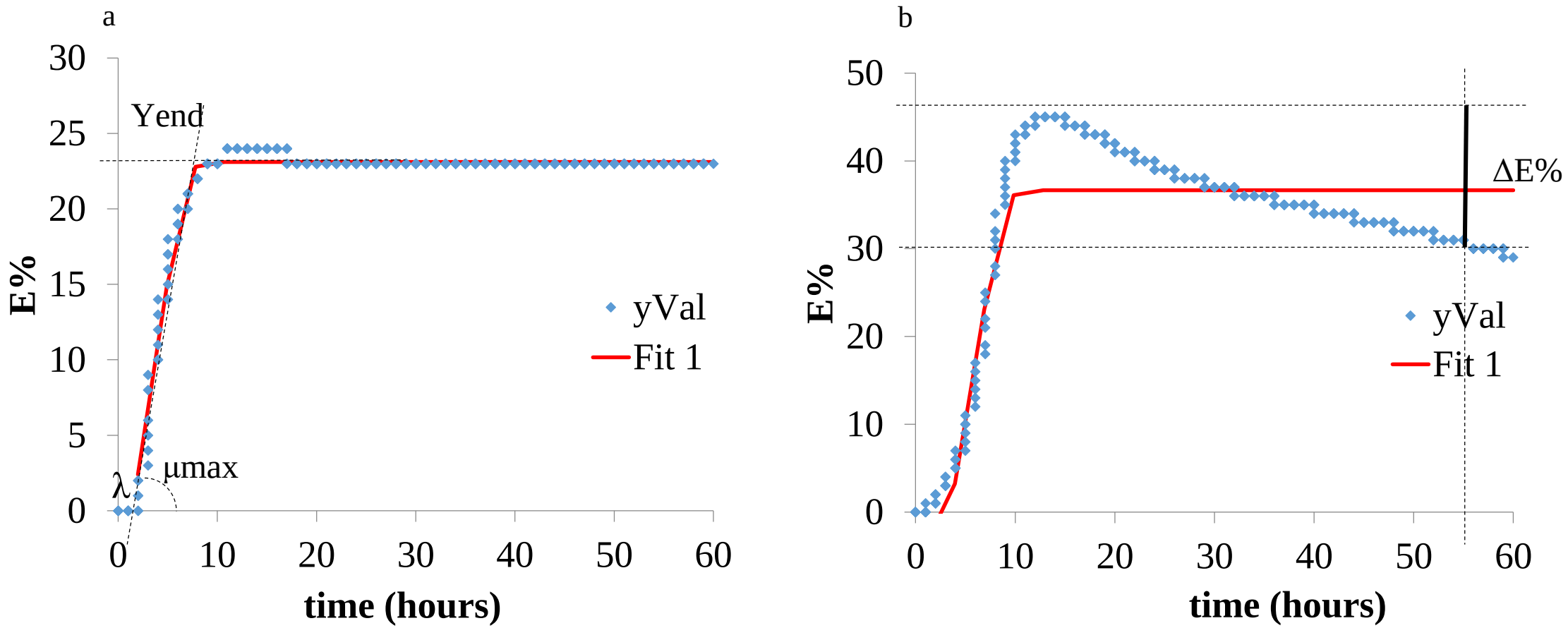

Figure 9

FIG 9. Schematic outline of the conversion mechanisms causing change of the capacitance curve (electrode impedance) E% at the four descriptive moments of LAB EPS- and LAB EPS+ strains growth in milk. Magnification of the electrode surface and of the molecules mainly involved in electrochemical phase at boundary metal/electrolytes: lactose (○), lactic acid (●) and EPS (◊).