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Different methodological approaches to study lactic acid bacteria traits for their potential technological application.

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*“Imagination is everything.
It is the preview of life's coming attractions.”*

A.E.

To my family

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Introduction

Lactic acid bacteria (LAB) have traditionally been associated with food fermentation due to their long and safe history of application and consumption in the production of fermented foods and beverages. For these reason some LAB strains have gained the Qualified Presumption of Safety (QPS) or Generally Recognized As Safe (GRAS) status (EFSA, 2010).

The main functionality of LAB in food fermentation processes is the rapid acidification of the food matrix. In addition, they are able to confer desirable attributes to specific fermented foods. Indeed, thanks to specific traits that they possess, they can form a wide range of compounds such as vitamins, bioactive peptides, aroma compounds and exopolysaccharides (EPS), that may positively impact the sensory quality of fermented foods (Leroy and De Vuyst 2004, Torino et al., 2015). Among the various application of LAB in the food processes, they are commonly used in dairy industries to acidify milk and to enhance the flavour of the end products.

In milk transformations LAB play an important role as starter (SLAB) in the acidification of milk, for fermented milk production, and in the acidification of curd, for cheese production. Furthermore, SLAB are involved in the flavour production that is a consequence of their secondary metabolisms and of the activity of their enzymes released after cells lysis. Non starter LAB (NSLAB), often used as secondary and adjunct starters for cheese production, can greatly influence flavour characteristics of the final product (Gobbetti et al., 2015). Another interesting ability of LAB in milk transformation is based on the of some strains is their ability to synthesize exopolysaccharides (EPS) that are polymers with an heterogeneous composition, structure and broad range of physicochemical properties and application (Malang et al., 2015, Galle et al., 2014). EPS show a positive effect on the texture, mouthfeel, taste perception and stability of fermented foods and they could be used to replace the use of external hydrocolloids derived from plant or seaweeds that are currently in use in food industries. Recently, the increasing demand for natural

polymers for industrial application, has led to a renewed interest in EPS production by LAB, especially for the in situ production (Leroy and De Vuyst 2004, Kumar et al., 2007, Zannini et al., 2015, Salvucci et al., 2016). As all the property of LAB is known to be strains dependent, the selection criteria both for SLAB and NSLAB result very important for choosing the right strain more suitable for the intended use.

Rationale and Aims

Considering all the desirable attributes that LAB can confer to food products, effort have been made to use LAB as microbial cell factories for the production of industrially interesting metabolites either to be used as purified compounds or to be produced *in situ* in fermented foods. (Torino et al., 2015, Bogouta et al., 2014, Galle et al., 2014). With the aim to select strains with interesting properties for milk products, in this PhD thesis, different methodological approaches has been used to study LAB to be used as new, functional cultures that may lead to an improved fermentation process and an enhanced quality of the end product.

- **Chapter 1** aimed to applied impedance microbiology to evaluate the potential acidifying performances of starter lactic acid bacteria to employ in milk transformation
- **Chapter 2** aimed to propose an integrated approach that could be employed for selection of the aromatic strains to be potentially used as adjunct starter in ripened cheese production.
- **Chapter 3** aimed to study the modification of the rheological characteristics of milk fermented by three EPS-producing lactic acid bacteria
- **Chapter 4** aimed to propose impedance microbiology as a screening methods to evaluate the exopolysaccharides in situ production by lactic acid bacteria

Chapter 1

Application of Impedance Microbiology for Evaluating Potential Acidifying Performances of Starter Lactic Acid Bacteria to Employ in Milk Transformation.

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1.1 Introduction

Impedance microbiology is a rapid method that enables qualitative and quantitative tracing of microorganisms by measuring the change in the electrical conductivity. It is based on a principle that dates back to 1899 (Stewart, 1899) but its application to food microbiology field is most recent and mainly associated to rapid detection of foodborne pathogenic bacteria (Yang and Bashir, 2007).

Impedance, applied to microbiology, can be defined as the resistance to flow of an alternating current that passes through a conducting microbial growth medium. During microbial growth, metabolic processes produce electrically measurable changes in the growth medium due to the metabolism of high-molecular weight nutrients into smaller charged ionic components that increase the electrical conductivity of the medium. Variation in electrical conductivity, monitored during time, is proportional to the change in the number of microorganisms and therefore the microbial growth can be measured (Batrinou et al., 2005).

Different systems, able to perform this measurement, are available. In the past, the most common commercial equipments used for impedance microbiology were, RABIT system™ (Don Whitley Scientific, Shipley, UK), Bactometer™ (bioMerieux, Marci l'Étolie, France) and Malthus™ (Malthus Instrument, Crawley, England). A more recent equipment is the BacTrac™ microorganism growth analyser (SyLab, Purkersdorf-Vienna, Austria). Common to all the systems is the measurement of an electronic signal that quantify the movement of ions between two electrodes (conductance) while, in some devices, the storage of charge at the electrodes medium interface (capacitance) is also measured (Noble et al., 1999). Plotting of the continuous measurement of cumulative increase in conductance, or capacitance, graphically results in an impedance curve (Rediers et al., 2012). The most common way to use this curve in microbiological analysis is fixing a point, generally defined as “time of detection”. However, fixed the microorganism, medium and

temperature, this point varies between one device and another. Time to detection (TTD) for RABIT corresponds to the point where the cumulative change in conductivity from the baseline meets or exceeds a set value over a defined time interval (Rediers et al., 2012). Detection Time (DT) for Malthus is obtained when a change in conductance over a threshold reference value set by the operator is observed (Lanzanova et al., 1993). DT of Bactometer is the amount of time required to cause a series of significant deviations from baseline impedance values (Noble et al., 1999). DT for Bactrac is the time when the impedance curve meets the threshold level of 5% (Čurda and Plocková, 1995). Indeed, the “time of detection” is the principal parameter measured by all the devices and it coincides with the reaching of a cells concentration of about 10^6 to 10^7 cells per ml (Noble et al 1999) thus, it is strongly affected by bacterial cells physiological state (Lanzanova et al., 1993). this parameter is largely used to monitoring pathogens or spoiling bacteria in food (Gracias and McKillip, 2004), and also antimicrobial activity (Silva et al., 2003; Marino et al., 2001; Kunicka-Styczyńska and Gibka, 2010) included lytic infections by bacteriophages (Amorim et al., 2009). Recently, an intriguing unconventional approach to impedance microbiology was considered to detect bacteriophages responsible for cell lysis (Mortari et al., 2015). However, to the author’s knowledge, the significance of the whole impedance curve have never been objectively related to microbial behavior.

The responses of microorganisms to specific environmental conditions, such as temperature, pH and a_w , can be described by predictive microbiology, a sub-discipline of food microbiology dealing with the development of mathematical models (Baranyi and Roberts, 1995). Several models have been developed to represent and predict microbial growth or inactivation in food and, nowadays, such models can be very useful in food technology and processing since they are applied to predict the outcome of fermentation processes under particular circumstances and to assess the effects of environmental conditions on microbial growth. Examples of primary models, widely applied to

describe the growth of lactic acid bacteria, include sigmoidal equations, such as Logistic and Modified Gompertz models (Chowdhury et al., 2007; Slongo et al., 2009). This describes the changes of the microbial population density as a function of time using a limited number of kinetic parameters (e.g., lag time, growth or inactivation rate and maximum population density) while it is not taken into account the stage of death. The Gompertz model provides a convenient mathematical tool that approximates the way in which microbiologists have traditionally estimated the graph of the growth kinetics (Buchanan et al., 1997).

Aim of this work was firstly to investigate the possibility to use the Gompertz equation to describe the data coming from the impedance curve obtained by mean of BacTrac 4300® and, secondly, to use the so described kinetics parameters, to evaluate the potential acidifying performances of several lactic acid bacteria strains for their possible use as starters in milk transformation.

1.2. Materials and methods

1.2.1 Strains, media and growth conditions.

Eighty strains representing four starter lactic acid bacteria species, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis* and *Streptococcus thermophilus* (Table 1), were analyzed by impedance measurements. The strains, belonging to the collection of the Laboratory of Food Microbiology of the Department of Food Science of University of Parma, have been previously isolated from dairy matrixes and identified by 16S rRNA sequencing.

Strains, maintained as frozen stocks cultures in MRS (Oxoid, Ltd., Basingstoke, United Kingdom) (*Lactobacillus*) or M17 (Oxoid Ltd.) (*Lactococcus* and *Streptococcus*) broth containing 20% (v/v) glycerol at -80 °C, were recovered in MRS or M17 broth by two overnight sub-culturing (5% v/v) at 42°C for *Lactobacillus* and *Streptococcus*, and 30°C for *Lc. lactis*. Then, other two eight hours sub-

culturing (5% v/v) of each strain in skim milk powder (Oxoid Ltd.), reconstituted to 10% (w/v) and sterilized at 110°C for 30 min (SSM), were performed before use.

1.2.2 Impedance measurement

A BacTrac 4300[®] Microbiological Analyzer (Sylab, Austria) system, consisted of two incubators allowing four different temperatures simultaneous setting, was used. The strains *L. helveticus* 5, *L. delbrueckii* subsp. *bulgaricus* 202, *Lc. lactis* 4068 and *S. thermophilus* 547 were 10-fold (first dilution), one hundred-fold (second dilution), one thousand-fold (third dilution), ten thousand-fold (fourth dilution), hundred thousand-fold (fifth dilution) diluted in ringer solution (Oxoid Ltd.). Not diluted culture and each dilution were inoculated (2% v/v) into previously sterilized measuring cells filled with 6 ml of SSM. The impedance measurement was performed at 42°C for *Lactobacillus* and *Streptococcus* strains, and 30°C for *Lactococcus* strains. Subsequently one hundred µl of the second dilution was used as inoculum for the analysis of all the 80 strains at their optimum growth temperature.

Moreover, three strains for each species (*L. helveticus* 3, 9, 23; *L. delbrueckii* subsp. *bulgaricus* 260, 265, 3436; *Lc. lactis* 664, 4064, 4067 and *S. thermophilus* 192, 160, 526) were also tested at different temperatures: 32°C, 37°C, 42°C, and 47°C for *Lactobacillus* and *Streptococcus* strains and 20°C, 25°C, 30°C and 35°C for *Lactococcus* strains. For each test, impedance measurement was recorded every 10 min for 80 hours. All the analysis were carried out in duplicated. One negative sample, consisting of non-inoculated SSM, was also incubated for each temperature tested.

1.2.3 Statistical analysis

The means and standard deviations of impedance changes in the medium (M%) data were calculated using SPSS (Version 21.0, SPSS Inc., Chicago, IL, USA) statistical software.

1.3. Results and discussion

1.3.1 Impedance curve interpretation

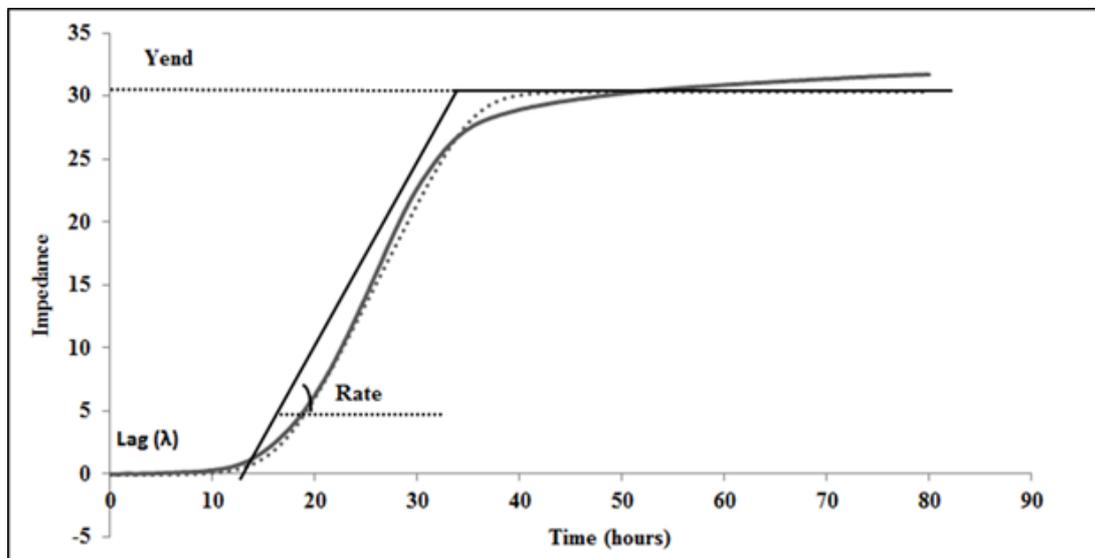
Impedance measurement is based on the principle that during microbial growth, metabolic processes produce electrically measurable changes in the growth medium. Milk has itself conductive properties because it is rich in charged compounds, especially minerals and salts (Mucchetti et al., 1994). During lactic acid fermentation, the decrease of lactose and the subsequent increase of lactic acid lower the medium pH and, at the same time, enhance its electrical conductivity as a result of the accumulation of lactate ions during fermentation (Carvalho et al., 2003).

Moreover, acidification of milk changes equilibria of buffer system and solubilizes casein-bound calcium and phosphorous salts. This phenomenon increases conductivity sharply, so there is a positive correlation between increased conductivity and milk acidification due to lactic acid bacteria activity.

This variation of electrical conductivity of milk is proportional to the change in microorganisms number and their metabolic activity and, therefore, microbial growth in milk can be measured (Mucchetti et al., 1994). The BacTrac 4300[®] system measures two specific impedance values, the E-value which is referred to as the impedance change at the electrode surface, and the M-Value which is the change in conductivity in medium, SSM in this case (Batrinou et al., 2005).

The system enables a separate registration of impedance changes in the SSM (M-value) and at the electrode (E-value). For the experiments carried out in the present study, the impedance change (M-value) of the SSM was used. This value, recorded every 10 minutes, is revealed as a relative change in the measurement signal and shown as M% percentage in function of time (80 hours) in an impedance curve (continues line in Figure 1).

Figure 1. A generalized impedance growth curve (continues line) and parameters extrapolated from curve by fitting data (dotted line): λ , lagtime; μ_{max} , maximum specific M% rate; Y_{end} , maximum value of M%.



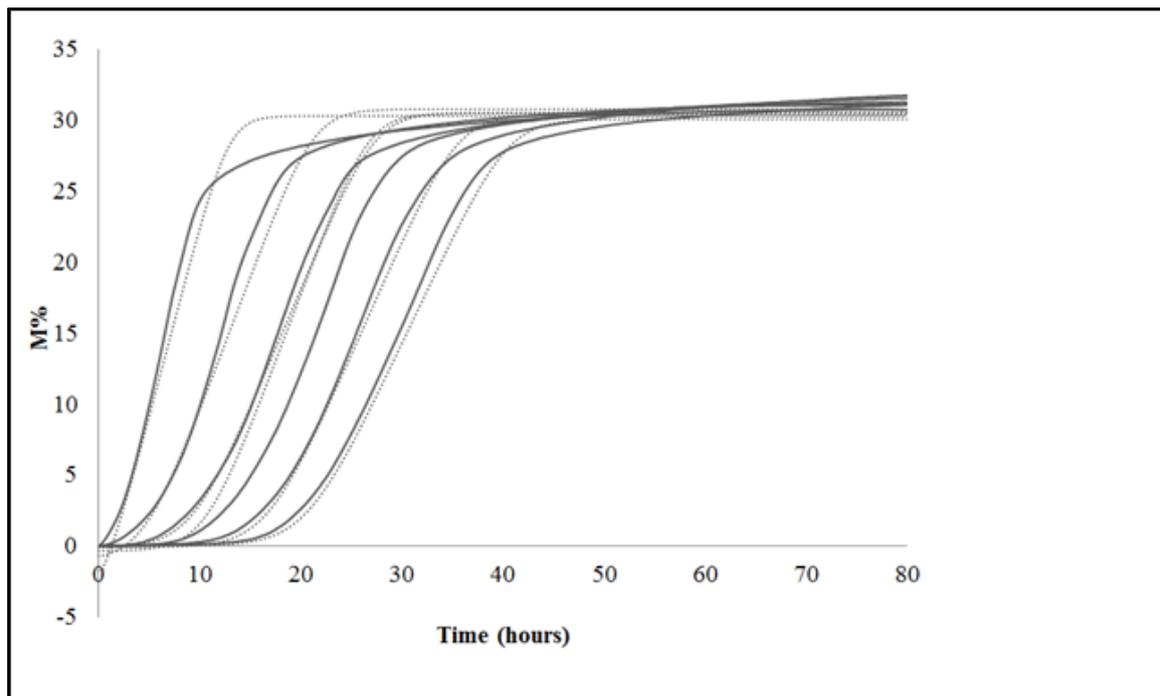
With the aim of translating the metabolic significance of the impedance curve into objective parameters, M% data were fitted to the Modified Gompertz equation (Gibson et al., 1988) using DMfit version 2.1 Excel add-in (<http://www.combase.cc/index.php/en/tools>). DMfit is part of the system used in-house at the Institute of Food Research to model the time-variation of the logarithm of cell concentration of bacterial batch cultures (www.ifr.ac.uk). Particularly, MS Excel adding DMfit is a free software application for predictive microbiology modelling developed by the Computational Biology Group at Institute of Food Research (Norwich, UK) (Perez-Rodriguez et al, 2013). Among the primary models available, modified Gompertz equation was used to describe the microbial evolution with time (Swinnen et al., 2004). In this research, the equation was used instead to describe M% in function of time. The fitted data are represented by a sigmoidal curve (shown as dotted line Figure 1) with two inflection points and generate 3 parameters: i) lag time (λ), ii) maximum specific M% rate (μ_{max}), and iii) maximum value of M% (Y_{end}) (Figure 1). The

possibility to fit the original data to the Modified Gompertz equation is tied to the necessity that the two curves overlap. All the curves obtained in this study have respected this rule (data not shown).

Lag phase is an adjustment period during which bacterial cells modify themselves in order to take advantage from the substrate, milk in this case, and initiate exponential growth, so the cells are assumed to be non replicating (Swinnen et al., 2004). The duration of the Lag phase depends on the strain, temperature and the substrate in which bacteria grow. Many hypotheses have been proposed to describe the formation and duration of the bacterial Lag phase in a growth curve. One of this hypotheses is the individual cell lag time theory (Huang, 2016). Based on this theory, the formation of Lag phase in a bacterial culture is determined by each cell and each cell may leave its lag state individually. Each cell would need to accumulate critical substance before it can grow and start dividing. Once a cell leaves its Lag phase, it enters the exponential phase, starting to grow and divide immediately (Huang, 2016). Based on this concept, lag time (λ) of an impedance curve can be considered as the time that the inoculated cells need to adapt to the condition of the analysis. In the same medium (SSM) at the same temperature (42° and 30°C depending on the species), as expected, for all the species the lower was the inoculum, the greater was the lag time and thus, this parameter is inoculum dependent (Table 2, Figure 2).

It has not been possible to register λ value for the inocula of the first and second dilutions of *S. thermophilus* because the time was incompatible with the *minimum* time of registration of the system that needs one hour to start recording data. During this time, λ values of the first and second dilutions are reached but not recorded.

Figure 2. Impedance curve (continuous line) and impedance curve obtained by fitting data (dotted line) of not diluted culture, first, second, third, fourth, and fifth dilutions.



The second parameter, maximum specific M% rate (μ_{max}) is comparable to the exponential phase and can be used to define LAB fermentation or acidification rate in SSM, which is an important parameter in technological processes, since the greater is the rate, the faster is the acidification. This parameter, excluding the undiluted inoculum for *L. helveticus* and *S. thermophilus*, was inoculum independent as evidenced by the coefficient of variation lower than 10% (Table 2).

Also for this parameter, the inocula with highest cell concentrations have a greater difficulty of using. Considering that the cells divide at a constant rate depending on the composition of the growth medium and the conditions of incubation, the M% rate (μ_{max}) parameter could also be used to determine the time of duplication or generation time.

Table 2. Values of Lag, Rate and y_{End} obtained from the serial dilutions of one strain for species.

Inoculum	<i>Lactobacillus helveticus</i> 5			<i>Lactobacillus delbrueckii</i> subsp <i>bulgaricus</i> 202			<i>Lactococcus lactis</i> 4068			<i>Streptococcus</i> <i>thermophilus</i> 547		
	λ (Lag)	μ_{max} (Rate)	y_{End}	λ (Lag)	μ_{max} (Rate)	y_{End}	λ (Lag)	μ_{max} (Rate)	y_{End}	λ (Lag)	μ_{max} (Rate)	y_{End}
Not diluted	0,68	2,64	30,06	1,12	2,33	25,63	1,20	4,25	25,14	nd [‡]	5,27	28,23
First*	4,58	1,97	30,55	3,29	2,76	26,98	2,62	4,04	25,27	nd [‡]	3,97	28,86
Second*	9,01	1,71	30,51	6,78	2,90	26,82	3,97	4,05	26,11	0,33	3,48	29,29
Third*	11,45	1,81	30,50	10,48	3,03	27,21	5,53	3,83	26,02	1,59	3,59	29,03
Fourth*	16,17	1,59	30,49	14,14	2,97	27,28	7,35	3,77	26,17	2,69	3,65	28,87
Fifth*	20,07	1,52	30,13	19,06	2,60	26,86	9,02	3,70	25,87	3,94	3,68	29,42
Mean	10,33	1,87	30,38	9,15	2,77	26,80	4,95	3,94	25,76	2,14	3,94	28,95
SD [§]	7,18	0,14	0,22	6,77	0,26	0,60	2,94	0,21	0,45	1,54	0,67	0,42
CV% [†]	69,56	21,77	0,72	74,05	9,51	2,24	59,39	5,25	1,73	72,20	17,00	1,44

[†] Coefficient of variation

[‡] not determined

* dilution

[§] Standard Deviation

However, as generation time is the time required for microbial cells to double in number (Madigan et al., 2009), to extrapolate the value of generation time from impedance value, a correlation with μ_{max} and number of cells should be carried out.

The third parameter (y_{End}), is the highest point of the fitted curve, very close to the maximum variation of impedance recorded (Figure 1). This value can be interpreted as the maximum capability of each strain to modify the impedance in SSM and thus depends mainly on its capability to accumulate lactate ions during growth. The capability to accumulate lactate ions can be measured as total amount of lactic acid, as for example, those produced in yoghurt, by means of different chemical methods (De Noni et al., 2004). The amount of lactate ions accumulated during growth depends on different aspects, among which, the initial amount of lactose, and acidity tolerance of the strains. In the same medium, with the same amount of initial lactose, y_{End} can be associated to the acidifying capability and to the resistance of the bacteria to the produced acidity.

Of the three considered parameters, Y_{end} is the more independent from the amount of inoculated cells (Table 2).

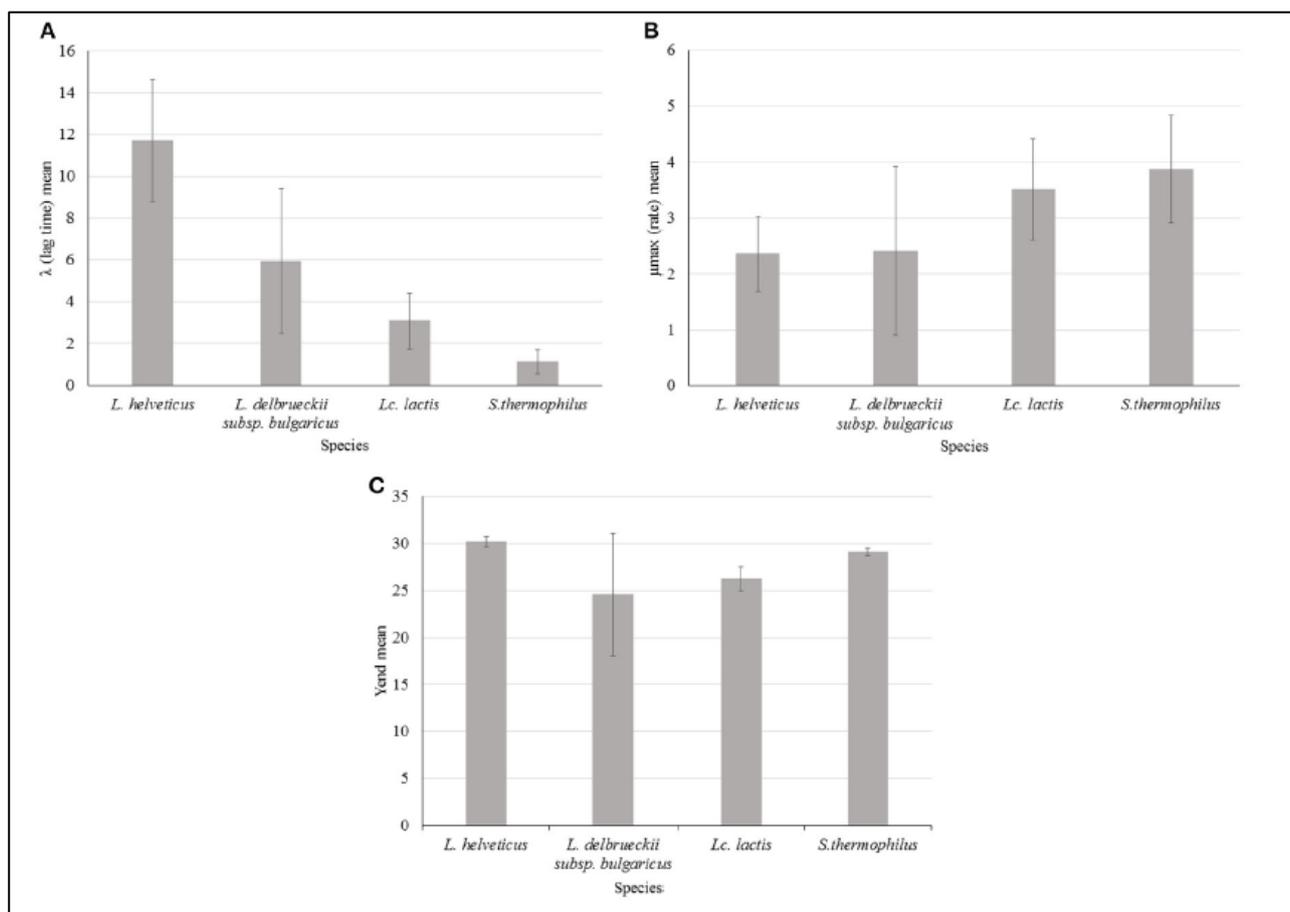
Considering what has been observed with this first part of the work, if the purpose is to know acidification rate and the amount of produced acid, different inoculum concentrations can be used, getting the same results. However, also considering the minimum time of registration of BacTrac 4300[®], the use of a high bacterial concentration, corresponding to the undiluted inoculum or to first dilution, has to be excluded, because it does not allow the visualization of the Lag phase. In this study, we wanted to consider also the λ value and thus we decide to carry out the analysis with the same inoculum concentration. The highest inoculum that allows the best description of the microbial growth performance in SSM was found to be the second dilution, that has been therefore used for the following determination.

1.3.2 Impedance analysis of starter LAB at optimal growth temperature

Aiming at evaluating the metabolic significance of the three kinetics parameters λ , μ_{max} and Y_{end} , 100 μ l of the second dilution of twenty strains for each considered LAB species were analyzed in duplicate at their optimal growth temperature, 42°C, for *Lactobacillus* and *Streptococcus*, and 30°C for *Lactococcus*.

λ was variable among species and *L. helveticus* showed, on average, the highest values of this parameter. This can be translated into a longer transition period during which the specific growth rate increases to the maximum value characteristic of the culture environment (Swinnen et al.,2004) and thus it can be interpreted as a slower adaptability of the species to the growth condition (Figure 3A).

Figure 3. Impedance analysis of four starter LAB species (*Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis*, and *Streptococcus thermophilus*) at optimal growth temperature: (A) λ (lag time) mean value of 20 strains for each species, (B) μ_{max} (rate) mean value of 20 strains for each species, (C) Y_{end} (maximum % M) mean value of 20 strains for each species.



Error bars show standard deviation (SD) for each species.

However λ was also highly variable within the species: *L. helveticus* and *L. delbrueckii* subsp. *bulgaricus* were the most heterogeneous species, as revealed by standard deviation values (SD) in Figure 3, while *Lc. lactis* and *S. thermophilus* strains showed the lowest and less variable values. Variability of *L. helveticus* in acidifying activity is well known and already measured in different way (Gatti et al., 2003). However, results coming from impedometric analysis and expressed as

time of detection, are prone to the variability of the used system. By using the λ value instead, measurements can be made and compared independently from the systems used for the analysis. Less used in food impedance microbiology but more interesting, if we consider its metabolic significance, is the parameter μ_{\max} . Thanks to the approach followed in this work, that is the elaboration of impedometric data by Modified Gompertz equation. It is possible to define and compare the different μ_{\max} features of the four species. Considering impedance curve of LAB in SSM, high μ_{\max} means high acidification rate and *S. thermophilus* and *Lc. lactis* showed higher value in milk respect to *Lactobacillus* species. To one side, this behavior of species is not new (Michel and Martley, 2001; Leroy and de Vuyst, 2004) but new and of great interest, is the possibility to easily compare acidification rates among the species and above all among the strains. In this regards, *L. delbrueckii* subsp. *bulgaricus* showed the highest intra-species variability (Figure 3B).

This variability could be of great technological interest because among the same species it is possible to choose the strain with higher or lower acidification rate depending on their possible application. For instance, fast acidifying ability can be required for a fermented milk production. On the other hand, lower rate could be desirable for mixed cultures where LAB coexist during changing environmental stimuli and stresses, which can affect their cellular physiology (Arioli et al., 2016).

Variability of maximum acidification rate, calculated measuring pH changing after defined time intervals, for 40 *S. thermophilus* strains, was already observed by Zanatta and Basso (Zanatta and Basso, 1992) using the system Micros (Conegliano, Italy). They found that the maximum acidification rate was the main variable discriminating strains in fast, medium and slow acidifying group (Zanatta and Basso, 1992). More similarly to our approach, a Don Whitley RABIT system (Sherry et al., 2006) was used for a qualitative study of *Salmonella*. μ_{\max} for 14 *Salmonella*

serovars was determined in less than 7 h, in respect to 24 hours needed by conventional method (Sherry et al., 2006).

Different values of Yend, thus different final acidification capability in milk, were found for the four species (Figure 3C). The highest value, with the lowest SD was found, on average, for *L. helveticus*. On the contrary, the lowest value, with the highest SD, was found for *L. delbrueckii* subsp. *bulgaricus*. This means that the first species has the best acidification capability, while the latter has the worst. However, *L. delbrueckii* subsp. *bulgaricus* also showed, as already observed for the others parameters, the more heterogeneous behavior within the species. This can be due to different acid resistance, but it can also be associated with the different capability of the species to metabolize the galactose moiety after lactose uptake. *L. helveticus* is able to ferment the glucose and galactose moieties of lactose (Mollet and Pilloud, 1991), while consumption of galactose by *L. delbrueckii* species depends on the subspecies, and the inability of the subspecies *delbrueckii* and *bulgaricus* to metabolize galactose could be due to the loss of the *galT* gene (Germond et al., 2003). The low average value of Yend and high level of SD measured for *L. delbrueckii* subsp. *bulgaricus*, were due to the presence of at least 6 strains characterized by Yend values lower than 20 (data not shown), possibly linked to the absence of the *galT* gene (Germond et al., 2003). Incapacity to metabolize galactose may be also the reason for lower levels of Yend found for *Lc. lactis* (Figure 3C), confirming that during the metabolism of lactose by *Lc. lactis*, part of the galactose 6P is dephosphorylated and excreted into the growth medium, while the glucose moiety is readily used (Neves et al., 2010). High and homogeneous level of Yend in *S. thermophilus* could be due to galactose positive strains. The existence of galactose negative strains has been reported, but only as a mutation of recent past (Vin et al., 2005).

Correlation between the two parameters μ_{max} and Yend was not found (data not shown), indicating that fastest strains were not always the greatest acidifying ones. Thus, this method of

characterization allow to choose the best strains considering which parameter is the most important for the desired technological application. For example, *L. helveticus* 35 was the best acidifying strain among all studied strains (Yend 31.4) but it was the slowest of its species (data not shown). On the other hand, one of the best acidifying *S. thermophilus* strain, 410 (Yend 29.6), was the faster (rate 5.8) among all studied strains (data not shown).

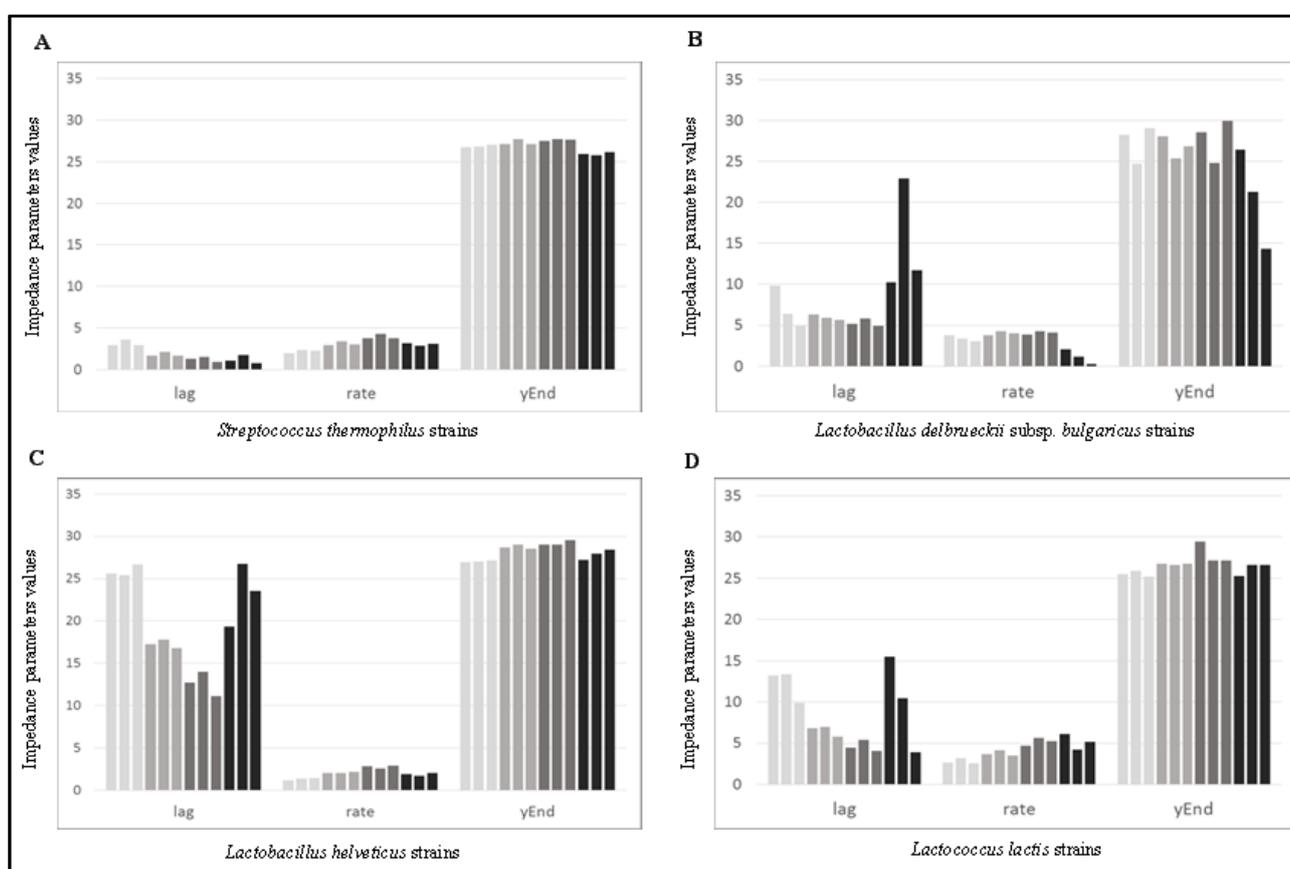
1.3.3 Impedance analysis of starter LAB at different growth temperature

Mesophilic bacteria, such as *Lc. lactis*, have an optimum growth temperature of 30°C, while thermophilic species, such as *L. helveticus*, *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, have an optimum growth temperature of 42°C. However, starter LAB employed in dairy fermentations can grow over a wide temperature range varying from 4 to 50°C (Hickey et al., 2015). This aspect is of particular importance because the milk transformations, such as microbial fermentation for the production of fermented milks and acidification of the curd in cheeses production, may involve temperatures quite far from the optimal for bacterial growth.

Considered this, in order to see how starter strains change their performances depending on temperatures, three strains for each species, chosen among the twenty previously evaluated, were tested through impedance analysis under temperatures 5°C and 10°C lower and 5°C higher than the optimal for thermophilic species and 5°C lower and 5° and 10°C higher than the optimal for mesophilic species. Varying temperature, Lag was the parameter that changed greatly. The differences were relevant for *L. delbrueckii* subsp. *bulgaricus*, *L. helveticus* and *Lc. lactis* and to a lesser extent for *S. thermophilus*, although it was clear that differences were strains dependent (Figure 4A, 4B, 4C, and 4D). The time of adaptation to the different temperatures was longer when temperature was higher than the optimal both for the *Lactobacillus* species and *Lc. lactis*. On the contrary, it was shorter, when temperatures were lower than optimal. However, the strains *L. helveticus* 3, *L. delbrueckii* subsp. *bulgaricus* 3436, and *Lc. lactis* 664 and 4067 showed to

adaptable more easily to the higher temperatures (Figure 4B, 4C and 4D). Of particular interest was the thermal tolerance observed for *L. delbrueckii* subsp. *bulgaricus* 3436 (Figure 4B).

Figure 4. λ (lag time), μ_{max} (rate) Y_{end} evaluated for (A) Three strains of *Streptococcus thermophilus* (192, 160 and 526) evaluated at 32°, 37°, 42°, and 47°C; (B) Three strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* (260, 265 and 3436) evaluated at 32°, 37°, 42°, and 47°C; (C) Three strains of *Lactobacillus helveticus* (3, 9 and 23) evaluated at 32°, 37°, 42°, and 47°C; (D) Three strains of *Lactococcus lactis* (664, 4064 and 4067) evaluated at 25°, 30°, 35°, and 40°C.



The acidification rate, measured as M% (μ_{max}), was variable for all species in function of the variation of the temperature; at the optimum, it was higher than rates at lower or higher temperatures for all strains (Figure 4A, 4B, 4C and 4D). This data is in agreement with the effect of changing temperature on the specific growth rate μ , evaluated by a pH-auxostat study for one

strain of *S. thermophilus*, one of *L. delbrueckii* subsp. *bulgaricus* and one of *Lc. lactis* (Adamberg et al., 2003). These authors observed that *S. thermophilus* had the highest specific growth rate at 44°C and a slight decrease at 45°C; 43°C was the temperature at which *L. delbrueckii* subsp. *bulgaricus* reached the maximum rate level, while *Lc. lactis* reached the maximum at 35°C and then, slightly decreased (Adamberg et al., 2003). Interestingly, in the present work, we found exceptions for *L. delbrueckii* subsp. *bulgaricus* 221, that at 47°C acidified faster than at its optimum (42°C) (Figure 4B).

All *Lc. lactis* strains, and in particular strain 4067, slowed consistently the rate when incubated at 40°C, while they tolerated the oscillation of 5 degrees, higher and lower than their optimum 30°C (Figure 4D).

The acidifying capacity was not greatly affected by the temperature for the thermophilic species even when incubated at 10°C below the optimum (42°C. Figure 4A, B and C). In particular, all the three *S. thermophilus* strains have maintained comparable acidification capacity values at each considered temperatures (Figure 4A). Instead, the response of *Lc. lactis* was strictly strain specific at all the temperatures. In particular, *Lc. lactis* 4067 and *Lc. lactis* 4064 showed similar acidification capacity at respectively 40°C and 30°C (optimum, Figure 4D).

In an intriguing experiment, it was demonstrated that one *Lc. lactis* strain, mutant TM29, after a long adaptation of 860 generation, was able to grow well up to 39°C due to mutations accumulated, most of which were shown to affect thermal tolerance (Chen et al., 2015) . The goal of that research was to demonstrate a simple approach to obtain non-GMO derivatives of *Lc. lactis* that possess properties desirable by the industry, such as thermal robustness and increased rate of acidification. In fact, Chen and colleagues (Chen et al., 2015) report that in the same cheese production, during the curdling process, the temperature is often raised to around 40°C, or even beyond, and in those condition *Lc. lactis* stops growing dramatically, reducing curd acidification. In

this perspective, *Lc. lactis* 4067, which by the way, is a wild strain isolated from raw cow milk used for Grana Padano cheese production, could have a great potential industrial interest.

1.4 Conclusion

The impedance microbiology is used since the seventies, but, besides the food control analysis to which it is commonly applied, only few researches had the purpose to study its different potential applications. With this work we wanted to find an objective way to interpret the metabolic significance of impedance curves and propose it as a valid approach to evaluate the potential acidifying performances of starter lactic acid bacteria to employ in milk transformation. The novelty and importance of our findings, obtained by means of BacTrac 4300[®], are that they can also be applied to data obtained from other impedometric devices. Moreover, the meaning of Lag, μ_{max} and Yend that we have extrapolated from modified Gompertz equation and discussed for LAB in milk, can be exploited also to other food environment or other bacteria, assuming that they can give a curve and that curve is properly fitted with Gompertz equation. Through this study, it was possible to highlight that the LAB species with the highest acidification rate were *S. thermophilus* and *Lc. lactis*, while *L. helveticus* and *S. thermophilus* showed the greatest acidification capacity. Among the eighty studied strains, twenty for each species, the widest heterogeneity was observed within *L. delbrueckii* subsp. *bulgaricus* subspecies. This intraspecific diversity was particularly evident when temperature was far from the optimal. Results obtained for some strains may be of interest for fermented milk and cheese production, particularly for cooked or semi-cooked cheeses.

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The authors are grateful to Sacco Clerici Group (22071 Cadorago, Italy) for making available some strains used in this research.

Table 1. Lactic acid bacteria strains used in this study.

Species	Strain	Source	Origin
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	260	Clerici-Sacco Group	Commercial starter
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	265	UNIPR	Natural whey starter
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	308	UNIPR	Natural whey starter
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	1865	UNIPR	Natural whey starter
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	1932	UNIPR	Curd
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	1982	UNIPR	Curd
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	2000	UNIPR	Curd
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	2225	UNIPR	Milk
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	2230	UNIPR	Milk
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	3436	UNIPR	Cheese
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	4622	UNIPR	Italian yogurt
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	4623	UNIPR	Italian yogurt
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	4624	UNIPR	Italian yogurt
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	4625	UNIPR	Italian yogurt
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	4626	UNIPR	Italian yogurt
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	4627	UNIPR	Italian yogurt
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	4628	UNIPR	Italian yogurt
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	4629	UNIPR	Italian yogurt
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	LMG 6901	NCIMB	Bulgarian yogurt
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	LMG 12168	NCIMB	Homemade yogurt
<i>Lactobacillus helveticus</i>	1	UNIPR	Natural whey starter
<i>Lactobacillus helveticus</i>	2	UNIPR	Natural whey starter
<i>Lactobacillus helveticus</i>	3	UNIPR	Natural whey starter
<i>Lactobacillus helveticus</i>	4	UNIPR	Natural whey starter
<i>Lactobacillus helveticus</i>	5	UNIPR	Natural whey starter
<i>Lactobacillus helveticus</i>	6	UNIPR	Natural whey starter
<i>Lactobacillus helveticus</i>	8	UNIPR	Natural whey starter
<i>Lactobacillus helveticus</i>	9	UNIPR	Natural whey starter
<i>Lactobacillus helveticus</i>	11	UNIPR	Natural whey starter
<i>Lactobacillus helveticus</i>	13	Clerici-Sacco Group	Commercial starter
<i>Lactobacillus helveticus</i>	23	UNIPR	Natural whey starter
<i>Lactobacillus helveticus</i>	28	UNIPR	Natural whey starter
<i>Lactobacillus helveticus</i>	35	UNIPR	Natural whey starter
<i>Lactobacillus helveticus</i>	36	UNIPR	Natural whey starter
<i>Lactobacillus helveticus</i>	37	UNIPR	Natural whey starter
<i>Lactobacillus helveticus</i>	39	UNIPR	Natural whey starter
<i>Lactobacillus helveticus</i>	41	UNIPR	Natural whey starter
<i>Lactobacillus helveticus</i>	42	UNIPR	Natural whey starter
<i>Lactobacillus helveticus</i>	1697	UNIPR	Natural whey
<i>Lactobacillus helveticus</i>	2457	UNIPR	Cheese
<i>Lactococcus lactis</i>	220	Clerici-Sacco Group	Commercial starter
<i>Lactococcus lactis</i>	225	Clerici-Sacco Group	Commercial starter
<i>Lactococcus lactis</i>	662	UNIPR	Milk
<i>Lactococcus lactis</i>	663	UNIPR	Milk
<i>Lactococcus lactis</i>	664	UNIPR	Milk
<i>Lactococcus lactis</i>	667	UNIPR	Milk
<i>Lactococcus lactis</i>	672	UNIPR	Milk
<i>Lactococcus lactis</i>	674	UNIPR	Milk
<i>Lactococcus lactis</i>	1239	UNIPR	Milk
<i>Lactococcus lactis</i>	1426	UNIPR	Milk
<i>Lactococcus lactis</i>	1428	UNIPR	Milk

Table 1. Continued

Species	Strain	Source	Origin
<i>Lactococcus lactis</i>	1439	UNIPR	Milk
<i>Lactococcus lactis</i>	2269	UNIPR	Milk
<i>Lactococcus lactis</i>	2270	UNIPR	Milk
<i>Lactococcus lactis</i>	2271	UNIPR	Milk
<i>Lactococcus lactis</i>	4062	UNIPR	Cheese
<i>Lactococcus lactis</i>	4064	UNIPR	Cheese
<i>Lactococcus lactis</i>	4065	UNIPR	Cheese
<i>Lactococcus lactis</i>	4067	UNIPR	Cheese
<i>Lactococcus lactis</i>	4068	UNIPR	Cheese
<i>Streptococcus thermophilus</i>	83	UNIPR	Milk
<i>Streptococcus thermophilus</i>	84	UNIPR	Milk
<i>Streptococcus thermophilus</i>	95	UNIPR	Curd
<i>Streptococcus thermophilus</i>	111	UNIPR	Milk
<i>Streptococcus thermophilus</i>	113	UNIPR	Curd
<i>Streptococcus thermophilus</i>	114	UNIPR	Curd
<i>Streptococcus thermophilus</i>	140	UNIPR	Curd
<i>Streptococcus thermophilus</i>	145	UNIPR	Milk
<i>Streptococcus thermophilus</i>	159	UNIPR	Milk
<i>Streptococcus thermophilus</i>	160	UNIPR	Milk
<i>Streptococcus thermophilus</i>	161	UNIPR	Milk
<i>Streptococcus thermophilus</i>	162	UNIPR	Milk
<i>Streptococcus thermophilus</i>	163	UNIPR	Milk
<i>Streptococcus thermophilus</i>	176	UNIPR	Curd
<i>Streptococcus thermophilus</i>	192	UNIPR	Milk
<i>Streptococcus thermophilus</i>	410	UNIPR	Milk
<i>Streptococcus thermophilus</i>	526	UNIPR	Milk
<i>Streptococcus thermophilus</i>	530	UNIPR	Curd
<i>Streptococcus thermophilus</i>	547	UNIPR	Curd
<i>Streptococcus thermophilus</i>	4028	UNIPR	Curd

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Chapter 2

An integrated strategy to discover *Lactobacillus casei* group strains for their potential use as aromatic starters

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2.1 Introduction

Flavour perception is very important in the distinction of fermented dairy products and for the determination of their quality (Olson, 1990). The combination of a large number of sapid volatile and non-volatile compounds, in the correct ratios and concentrations, leads to the formation of cheese flavour (Mc Sweeney and Sousa, 2000) that is one of the most important criteria in determining consumer choice and acceptance. Lactic acid bacteria (LAB) manages, through the metabolic conversion of precursors, the production of volatile compounds that are related to specific aroma. LAB that contribute to cheese process may include deliberately added starters (natural or selected acidifying culture), adjunct bacteria (selected strains that intensify or accelerate flavour development) and adventitious species, which may enter the cheese making process through the milk, the cheese making equipment and/or the production environment (Broadbent et al., 2005).

These bacteria collectively influence flavour development through several basic mechanisms that include 3 major metabolic pathways: (1) metabolism of lactate and citrate (Mc Sweeney, 2004; Thierry et al., 2015), (2) liberation of free fatty acids (FFA) and their subsequent metabolism (Collins et al., 2003), (3) degradation of the casein matrix of the curd to a range of peptides, which is followed by degradation to free amino acids (AA), and ultimately involves the catabolism of free AA (Fernández and Zúñiga, 2006; Mc Sweeney and Sousa, 2000; Yvon and Rijnen, 2001).

The study of these metabolic traits is particularly essential to choose the strains with aroma potential, which could be used to modify or enhance cheese flavour. A careful screening of potential adjunct strains must be done before their introduction in large-scale fermentation models (Banks and Williams, 2004; Tanous et al., 2002; Van Hoorde et al., 2010; Williams et al., 2000). Furthermore, dairy companies have increased their effort to extend culture collections with strains isolated from diverse origins to steer aroma formation in fermented dairy products (Smid

and Kleerebezem, 2014). As recently suggested, much of the future efforts should be addressed to search for strains which should exploit the potential for cheese ripening (Gobbetti et al 2015). Differently from acidifying starters, adjunct cultures should be able to reach and maintain high numbers during ripening without affecting the cheese manufacturing process. Thus, the selection of strains has to be done taking into account the technological properties relevant for cheese making such as acidifying activities (Randazzo et al., 2007).

Considering their main role, among different LAB species, especially *Lactobacillus casei* group (Stefanovic et al., 2017), can be selected and used as adjunct starter. However, their potential ability to develop and maintain viability in non-optimal condition, such as cheese ripening, has to be considered. Recently, a method to screen for potentially interesting LAB adjunct strains, *L. paracasei*, was reported to the aim of use adjunct cultures in the production of new types of artisanal Gouda-type cheeses (Van Hoorde et al., 2010). However, most of these strains were not directly isolated from the milk, but from the different step of cheeses production (Van Hoorde et al., 2008, Gobbetti et al 2015). This species, that together with *L. casei* and *L. rhamnosus* forms the *L. casei* group, characterizes also the microbiota of highly appreciated long ripened Parmigiano Reggiano cheese and is involved in aroma production (Gatti et al., 2014). The origin of these species is raw milk, as previewed in the specification of the Protected Designation of Origin (PDO) (http://www.parmigianoreggiano.com/consortium/rules_regulation_2/default.aspx). However, in raw milk, *Lactobacillus* genus is only minority respect to the complex microbiota (Addis et al., 2016; Quigley et al., 2013). The aim of this research is to find a strategy to isolate, directly from milk, new strains able to produce aroma compounds, potentially useable in long ripened cheese production as adjunctive aromatic starters. To reach this goal, a spontaneous fermentation of raw milks used to produce Parmigiano Reggiano cheese was carried out at 8°C for 4 months. Specific selection criteria were chosen to isolate strains belonging to *L. casei* group, from raw milk where

they are minority. An integrated approach, by means of impedance microbiology and headspace solid-phase microextraction followed by gas chromatography–mass spectrometry (SPME GC-MS) analysis, was applied to investigate the acidifying performance and the production of volatile compounds of seven strains in ultra-high-temperature (UHT) whole milk. This approach could be employed in the selection of strains to be used as adjunct starters in different dairy applications.

2.2 Material and Methods

2.2.1 Sample collection

A total of 14 samples of raw cow milk were collected from different Parmigiano Reggiano cheese factories in the province of Parma. The milk samples were collected in sterile bottles, transported to the laboratory in a cool box and stored at 8°C for 4 months. Two out of 14 samples were selected, basing on the most appreciated aroma perceived, for the following steps.

2.2.2 de Man, Rogosa and Sharpe plate count

The 10^{-1} dilutions of the two chosen fermented milk samples, were made by diluting 10 ml of milk with 90 ml of Ringer's solution (Oxoid, Ltd., Basingstoke, United Kingdom). Further, tenfold serial dilutions, ranging from 10^{-2} to 10^{-7} , were plated on de Man, Rogosa and Sharpe (MRS) agar (Oxoid), incubated at 30°C for 48 hours anaerobically. Several colonies were randomly picked from the plates of the highest dilutions privileging the different morphology. They were purified by sub-culturing on fresh agar plates of the isolation medium followed by microscopic examination.

Sixty-three purified colonies were maintained as frozen stocks cultures in MRS broth containing 20% (v/v) glycerol at -80°C.

2.2.3 Identification of *Lactobacillus casei* group and species

To identify the belonging to *L. casei* group, the 63 purified colonies were analysed by colonies PCR, using specific primers *poxcDNAFw* CAGACGCAATGATCAAGGTG and *poxPromRv* AATGCGCCyACTTCTTCATG, to identify strains belonging to *L. casei* group (Savo Sardaro et al., 2016). The colonies were analysed by using PCR reactions composed of 8 µL of sterile MilliQ water, 10 µL of 2XPCR GoTaq Master Mix (Promega), 1 µL of forward primer (10 mM), 1 mL of reverse primer (10 mM) and colonies template DNA following the authors thermal cycling conditions. The presence of the amplification products were evaluated by 1.5% agarose gel electrophoresis.

To identify the species, two different identification methods were used, 16S rRNA gene sequences and High-Resolution Melting (HRM) analysis (Savo Sardaro et al., 2016). Genomic DNA was extracted from the isolates cultured overnight, using the DNeasy Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The DNA concentration was measured using an Eppendorf BioPhotometer Plus instrument and checked by agarose gel electrophoresis. Genomic DNA was diluted to 20 ng/µL for PCR. The bacterial 16S rRNA gene was amplified from the total genomic DNA using universal specific primers 63F (5'CAGGCCTAACACATGCAAGTC 3') and 1387R (5'GGGCGGAGTG TACAAGGC 3'), which yielded a product of approximately 1300 base pairs (Marchesi et al., 1998). The PCR conditions were 30 cycles of 95 °C denaturation for 1 min, annealing at 55 °C for 45 s and extension at 72 °C for 1 min and in addition one cycle of extension at 72 °C for 7 min. The amplified DNAs were sent to BMR Genomics (Padova, Italy) for sequencing. The taxonomic strain identification was performed at the Blastn site of NCBI server.

HRM analysis by PCR was performed by using the previous primers *poxcDNAFw* and *poxPromRv* using QuantStudio 3 Real-Time PCR System thermal cycler (Life Technologies, Carlsbad, USA) following the protocol described by Savo Sardaro et al., 2016. The seven strains were tested in triplicate and non-template controls were included in each run.

2.2.4 Milk coagulation analysis

Only the isolates belonging to *L. casei* group were cultivated, and inoculated (2% v/v) in skim milk powder (Oxoid Ltd.), reconstituted to 10% (w/v) and sterilized at 110°C for 30 min (SSM). Cultures were incubated at 37°C in aerobic condition for 5 days. Coagulum formation has been visually evaluated after 6, 12, 24 hours, 2, 3, 4, and 5 days. Basing on how long it has been necessary before to visualize the clot, strains were grouped into four classes.

2.2.5 Acidifying performance analyses

Seven identified strains, chosen for the evaluation of acidifying performance and the production of volatile compounds, were cultured in MRS broth by two overnight sub-culturing (2% v/v) at 37°C, followed by an overnight sub-culturing in UHT whole milk. After incubation in aerobic conditions at 37°C, each culture was inoculated (2% v/v) in 5 ml of UHT whole milk. The cultures were inoculated (2% v/v) into previously sterilized impedance measuring cells filled with 6ml of UHT whole milk. Impedance measurements were performed by using a BacTrac 4300® Microbiological Analyzer (Sylab, Austria) system at 37°C (Bancalari et al., 2016). The analysis was carried out in triplicated and impedance measurement was recorded every 20 minutes for 5 days. One negative sample, consisting in non-inoculated UHT whole milk, was also evaluated. pH was measured after 5 days by means of pH meter Beckman Instrument mod Φ 350 (Furlenton, CA, USA) and Hamilton glass electrode (Bonaduz, Switzerland).

2.2.6 Analysis of the volatile compounds

The seven selected *L. casei* group strains were also inoculated in 5 ml of UHT whole milk directly in vials for SPME GC-MS analysis. The analysis was carried out in triplicated and the volatile compounds produced by the seven strains grown in UHT whole milk for 5 days, were detected through SPME GC-MS analysis of the headspace of the sealed vials in which they were grown.

Before analysis, 5 μl of toluene solution (250 mg/L in water) as internal standard (Sigma, St. Louis, MO, USA) were inserted in the sealed vials perforating the vial septum with a syringe.

GC-MS analysis was performed using a TRACE 1300 gas chromatograph (Thermo Fisher Scientific Inc.) coupled to a ISQ mass spectrometer (Thermo Fisher Scientific Inc.). An SPME fiber assembly with a divinylbenzene-carboxen-polydimethylsiloxane coating 50/30 μm (Supelco Inc., Bellefonte, PA, USA) was used after preconditioning according to the manufacturer's instruction manual. The samples were preheated for 10 min at 40°C (equilibration step) and then the SPME fiber was exposed to each sample for 20 min at 40°C (extraction step). The vial was maintained at 40°C under magnetic stirring. Finally the fiber was inserted into the injection port of the GC for 3 min of sample desorption at 250 °C. A Zebron ZB-FFAP capillary column (Phenomenex, CA, USA, 30 m \times 0.25 mm, f.t. 0.25 μm) was used. Volatile compounds were separated under the following conditions: helium carrier gas (1 mL/min), an initial column temperature of 40°C for 3 min, an increase of temperature to 200°C at 5°C/min and maintenance at 200°C for 5 min. Each chromatogram obtained contained a high number of peaks which were identified based on three criteria: (1) by comparing the mass spectra with the NIST14 library of mass spectra (2) by comparing the retention index with literature data, and (3) whenever possible, the identification was confirmed by using pure standards of the components. Retention indexes were calculated using n-alkanes (C8-C17) (Sigma –Aldrich, Milan, Italy) as reference compounds according to Bianchi et al., (2007). In addition, semi-quantitative information was derived by comparing the relative peak areas of the volatiles to the peak area of toluene (the internal standard). Preliminary trials for UHT milk stored for 5 days at 37°C without inoculum were performed but no differences with the control were observed. For each strain, the volatile content of the blank (UHT whole milk used as substrate for the inoculum) was subtracted to the volatile content of the cultures. In this

way, the volatile compounds identified and quantified are specifically related to the strains metabolism.

2.2.7 Statistical analysis

Volatile compounds, impedance parameters and pH detected for each strain are presented as mean of the three different samples. For each parameter considered, one-way ANOVA and post hoc Tukey-HSD tests (p level 0.05) were performed to detect significant differences among the volatile production and acidifying ability of the seven strains. Correlation coefficient among pH and acidifying parameters was evaluated by bivariate Spearman's correlation using IBM SPSS statistics package version 21 (IBM Corp., Armonk, NY, USA). Principal component analysis (PCA), carried out on all the data collected, to describe the variation in the dataset and highlight the differences among strains was performed utilizing the statistical package Statistica 6.1 (StatSoft Italy srl, Vigonza, Italy)

2.3 Results and discussion

2.3.1 From the spontaneous fermentation to strains selection

The aim of this research was to find a strategy to isolate, directly from milk, new strains able to produce aroma compounds, potentially useable in long ripened cheese production as adjunctive aromatic starter. We focused on species that are known to be involved in the production of end metabolites that contribute to flavour development such as *L. casei*, *L. paracasei* and *L. rhamnosus*. Members of these species, belonging to *L. casei* group, are facultative heterofermentative bacteria which are known to tolerate the conditions encountered in ripened cheese environment, such as nutrients depletion, low pH and low temperature (Salveti et al., 2012, Gatti et al., 2014, Gobbetti et al., 2015). Raw cows' milk contain diverse bacterial population, consisting of psychrotolerant microorganisms, mainly belonging to the genus *Pseudomonas*, as well as other species from the

family of *Lactobacillaceae*, such as the genus *Lactococcus* or *Lactobacillus* (Ercolini et al., 2009; Vacheyrou et al., 2011; Quigley et al., 2013). In this environment, *Lactobacillus* genus is generally present at low concentrations (Addis et al., 2016). Even less, the *L. casei* group concentration. On the other hand, it is well known that these species are largely involved in the aroma formation of highly appreciated Italian long ripened (Gatti et al., 2014). For this reason, in this study, we chose to perform a long spontaneous milk fermentation at low temperature in order to highlight and select these bacteria.

Preliminarily, 14 raw milk samples coming from different Parmigiano Reggiano cheese factories were spontaneously fermented at 8°C for 4 months and after this period two milk samples were chosen for their appreciated aroma perceived (data not shown).

Thirty-nine of the 63 colonies picked from the MRS plates of the highest dilutions evidenced the presence of 150 bp in the gel (data not shown) indicating belonging to *L. casei* group (Savo Sardaro et al., 2016).

In order to further select the strains to use for the following analysis the different ability to coagulate milk was tested. Milk coagulation was monitored after 6, 12 and 24 hours, and successively one time a day for five days. All the 39 *L. casei* group strains have caused milk coagulation after 2 days of incubation. However, the coagulation time was variable and strain-dependent. The strains were grouped according to the time required to coagulate milk in four classes. In particular, 21% of the strains were able to coagulate milk in 6 hours, 38% in 12 hours, 23% in 24 hour and the remaining 18% after 2 days of incubation (Table S1). Considering the different time of coagulation, one strain for classes I, III and IV and four strains for most frequent class II, were chosen for the following characterisation (Table 1).

Table 1. Ability to coagulate skim milk of the seven *L. casei* group strains. Visualization of coagulum formation after different times

Strains	Time of incubation							classes
	6 hours	12 hours	24 hours	2 days	3 days	4 days	5 days	
4339	0*	0	1**	1	1	1	1	III
4340	0	1	1	1	1	1	1	II
4341	0	0	0	1	1	1	1	IV
4343	1	1	1	1	1	1	1	I
4357	0	1	1	1	1	1	1	II
4360	0	1	1	1	1	1	1	II
4366	0	1	1	1	1	1	1	II

* liquid milk



** coagulate milk



2.3.2 Strains identification

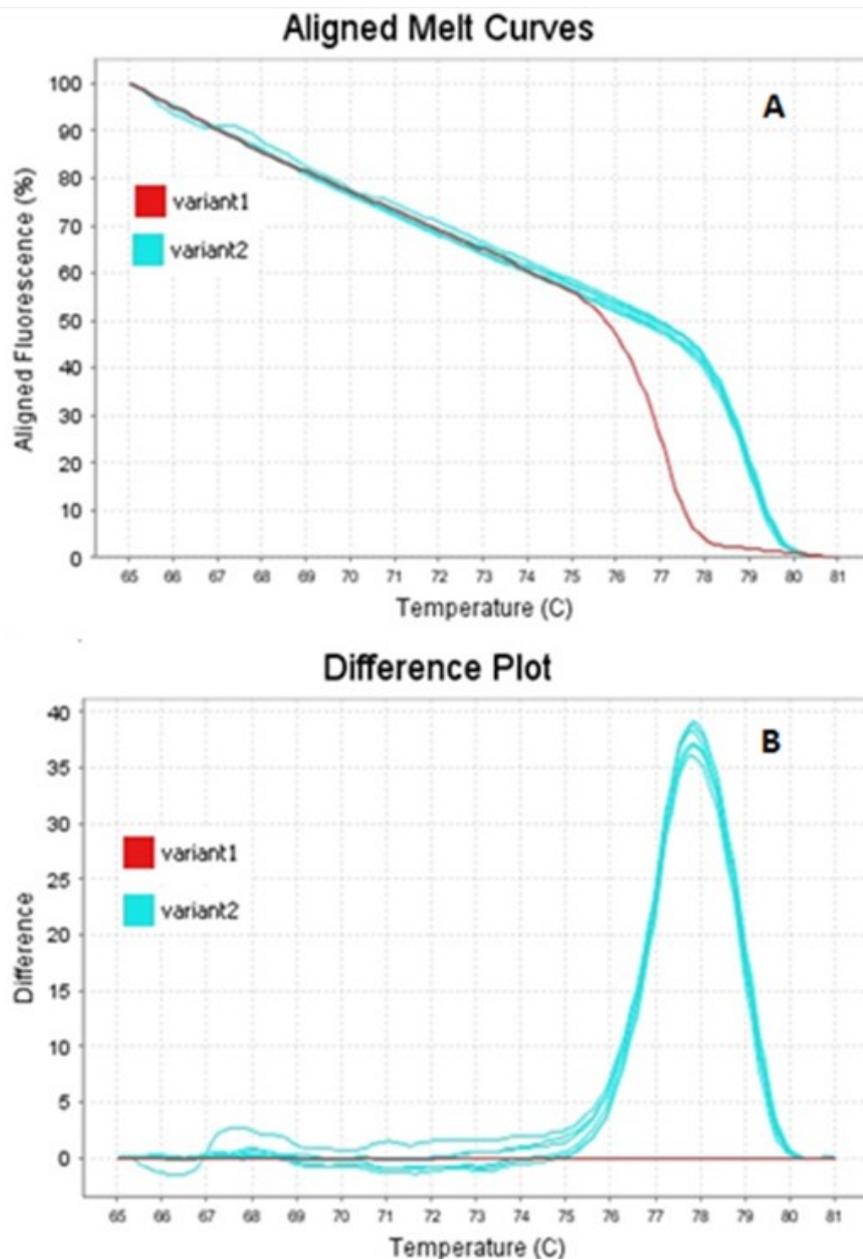
As the species belonging to the *L. casei* group, *L. casei*, *L. paracasei* and *L. rhamnosus* are phylogenetically and phenotypically related and therefore difficult to discriminate (Sun et al., 2015), two methods of identification were used. First of all, the seven strains were evaluated by 16S sequences analysis. The results shown in Table 2 highlighted that the seven strains have close sequence similarity with both *L. casei* and *L. paracasei* with the same identity percentage and therefore it was not possible to define the specific subspecies.

Table 2. Strains identification

Strains	16S sequences closest relative species	Gene Bank Acc. No.	Identity (%)	HRM methods
4339	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i> strain 46-213	HQ697655.1 KY471695.1	99 99	<i>Lactobacillus casei</i> (putative)
	<i>Lactobacillus casei</i> strain B9-1			
4340	<i>Lactobacillus paracasei</i> S602	KX057654.1	100	<i>Lactobacillus</i>
	<i>Lactobacillus casei</i> strain b92	KX303809.1	100	<i>paracasei</i>
4341	<i>Lactobacillus paracasei</i> strain BCH-5	KX388388.1	98	<i>Lactobacillus</i>
	<i>Lactobacillus casei</i> strain IMAU70037	GQ131153.1	98	<i>paracasei</i>
4343	<i>Lactobacillus paracasei</i> strain LP 244	KR006302.1	97	<i>Lactobacillus</i>
	<i>Lactobacillus casei</i> strain 7A04A1	KJ764639.1	97	<i>paracasei</i>
4357	<i>Lactobacillus paracasei</i> strain LP 242	KR006301.1	99	<i>Lactobacillus</i>
	<i>Lactobacillus casei</i> strain IMAU70037	GQ131153.1	99	<i>paracasei</i>
4360	<i>Lactobacillus paracasei</i> strain LP 244	KR006302.1	99	<i>Lactobacillus</i>
	<i>Lactobacillus casei</i> strain NG5 16S	KR107062.1	99	<i>paracasei</i>
4366	<i>Lactobacillus paracasei</i> strain BCH-5	KX388388.1	99	<i>Lactobacillus</i>
	<i>Lactobacillus casei</i> strain MI4SA5b1	KY287779.1	99	<i>paracasei</i>

For this reason, the HRM method for the seven strains discrimination was used. The amplification and dissociation curves of the seven strains isolated were analysed using the HRM Software v 2.0.1 (Life Technologies). The aligned melt curves (Fig.1A) and the difference plots (Fig.1B) obtained by using the fluorescence value of each strain allowed them to be grouped into two variants with a confidence level higher than 90%. Variant 1 contained the strain 4339, with T_m of $77^\circ \pm 0.1$ C, represented strain putative belonging to *L. casei* species and the Variant 2 that contained the others six *L. casei* group strains belonged to *L. paracasei* species, with an average T_m of 78.9 ± 0.1 °C.

Figure 1. High resolution melting analysis of the 150 bp *spxB* DNA fragments amplified by real-time PCR from the seven *L. casei* group strains. The aligned melt curves (A) and the difference plots (B) reported the two variants identified. Variant 1 (red) indicates *L. casei* strains; variant 2 (blue) indicates *L. paracasei* strains.



2.3.3 Analysis of the acidifying features of the selected strains

The acidifying features of the seven strains were evaluated by growth in UHT whole milk for five days. Recently, Bancalari et al. (2016) employed the impedance microbiology to trace microbial

growth in milk. The kinetics parameters Lag, Rate, yEnd were used to describe the potential acidifying performances. The Lag parameter, describing the time that the inoculated cells need to adapt to the growth conditions, was variable among the strains. In particular, as reported in Table 3, *L. paracasei* 4343, *L. paracasei* 4360 and *L. paracasei* 4366 showed the lowest Lag value, that can be explained as a faster adaptability in milk. On the other hand, *L. paracasei* 4341 was the slowest to adapt to milk, compared with the other strains. The lowest value of Rate, explained as acidification rate, was found for *L. paracasei* 4343 (fast acidification) and the highest for *L. paracasei* 4366 (low acidification). Finally, the last parameter, yEnd (describing acidifying capacity), resulted variable but not significantly different among the strains tested. After 5 days, at the end of impedance microbiology analysis, also the final pH was measured and a negative correlation ($r = -0.84$) was observed between pH and yEnd value (Table 3).

Table 3. Main values of the acidifying parameters obtained from impedance analysis and pH evaluated in triplicate for the seven selected strains

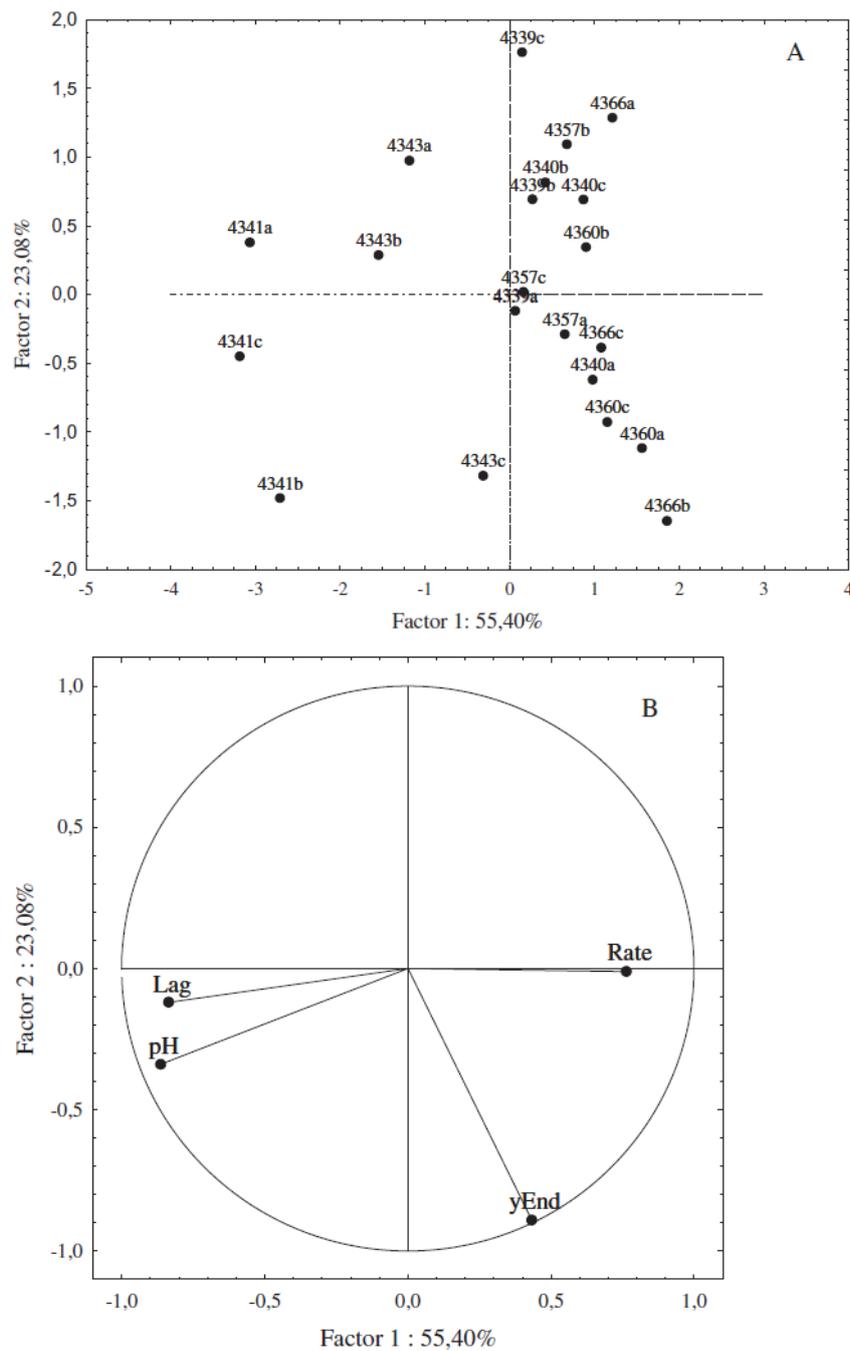
Strain	Impedance microbiology parameter			pH
	Lag*	Rate**	yEnd***	
Putative <i>L. casei</i> 4339	18.76±1.25	0.56±0.05 ^c	27.36±2.88 ^a	3.85±0.02 ^a
<i>L. paracasei</i> 4340	11.93±1.42 ^b	0.60±0.02 ^c	29.11±2.59 ^a	3.85±0.06 ^a
<i>L. paracasei</i> 4341	58.34±2.48 ^d	0.46±0.04 ^{a,b}	27.40±3.02 ^a	4.25±0.03 ^b
<i>L. paracasei</i> 4343	7.56±0.77 ^a	0.40±0.02 ^a	28.38±4.18 ^a	4.10±0.08 ^b
<i>L. paracasei</i> 4357	9.51±0.98 ^{a,b}	0.52±0.05 ^{b,c}	29.23±2.04 ^a	3.85±0.06 ^a
<i>L. paracasei</i> 4360	7.30±0.56 ^a	0.72±0.01 ^d	31.20±2.57 ^a	3.95±0.05 ^a
<i>L. paracasei</i> 4366	7.91±0.72 ^a	0.73±0.05 ^d	30.60±4.36 ^a	3.90±0.06 ^a

To better highlight the differences among the strains, an exploratory data analysis such as PCA was carried out using the kinetics parameters and pH values reported in Table 3.

The PCA loading plot on the 2 first factors relative to the seven strains cultivated in triplicate in milk is reported in Figure 2. Interestingly, the variables are well divided in the plot and Lag and pH

are directly correlated and give the same information in the discrimination of the strains (Figure 2a). The triplicate of the seven strains are distributed along both the principal component even if *L. paracasei* 4341 is better separated from the other strains for its higher value of Lag and pH.

Figure 2. Plots of loading (A) and score (B) obtained by PCA analysis of the pH and acidifying kinetic parameters of the 7 strains cultivated in triplicate (a,b,c) in milk.



2.3.4 Analysis of the volatile compounds produced by the selected strains

The study of the aromatic properties of the selected strains offers good possibilities to extend the flavour and diversity of dairy products. For this reason, the volatile compounds produced by the 7 strains grown in UHT whole milk for 5 days were detected through SPME GC-MS analysis. In the global volatile profile of inoculated milks, 16 compounds were found as specific products of the strains metabolism, these are reported in Table 4 together with their identification, odour characteristics and quantitative amounts.

The overall aromatic ability of the strains was compared taking into account the average total volatile compounds production (the sum of the mean values of the various classes of compounds). The total volatile compound production for each strain ranged between 25.63 µg/100ml to 110.13 µg/100ml. The highest concentration of total volatiles was found in milk fermented with *L. paracasei* 4341, while the lowest in milk fermented with putative *L. casei* 4339 (Table 4).

The volatile compounds identified were divided according to the chemical classes, and the following were identified: acids, ketones, alcohols, aldehydes and hydrocarbons. In particular, in the fermented milk samples the amount of acids ranged between 19,86 µg/100ml (putative *L. casei* 4339) and 67.44 µg/100ml (*L. paracasei* 4341); ketones/aldehydes were comprised between 2.65 µg/100ml (putative *L. casei* 4339) and 32.45 µg/100ml (*L. paracasei* 4341); alcohols ranged between 1.12 µg/100ml (*L. paracasei* 4343) and 9.70 µg/100ml (*L. paracasei* 4341); hydrocarbons were ranged between 0.51 µg/100ml (putative *L. casei* 4339) and 1.29 µg/100ml (*L. paracasei* 4360) (Table 4). *L. paracasei* 4341 and putative *L. casei* 4339, which had similar kinetic parameters, showed a significant difference ($p < 0,05$) for the Lag value, and a substantial difference in terms of total volatile compounds production (Tables 3 and 4). Analysing the different classes of aromatic compounds, *L. paracasei* 4341 differs mainly for the amount of acids, ketones/aldehydes and alcohols. In particular, among these compounds, this strain showed a significantly higher ($p < 0,05$)

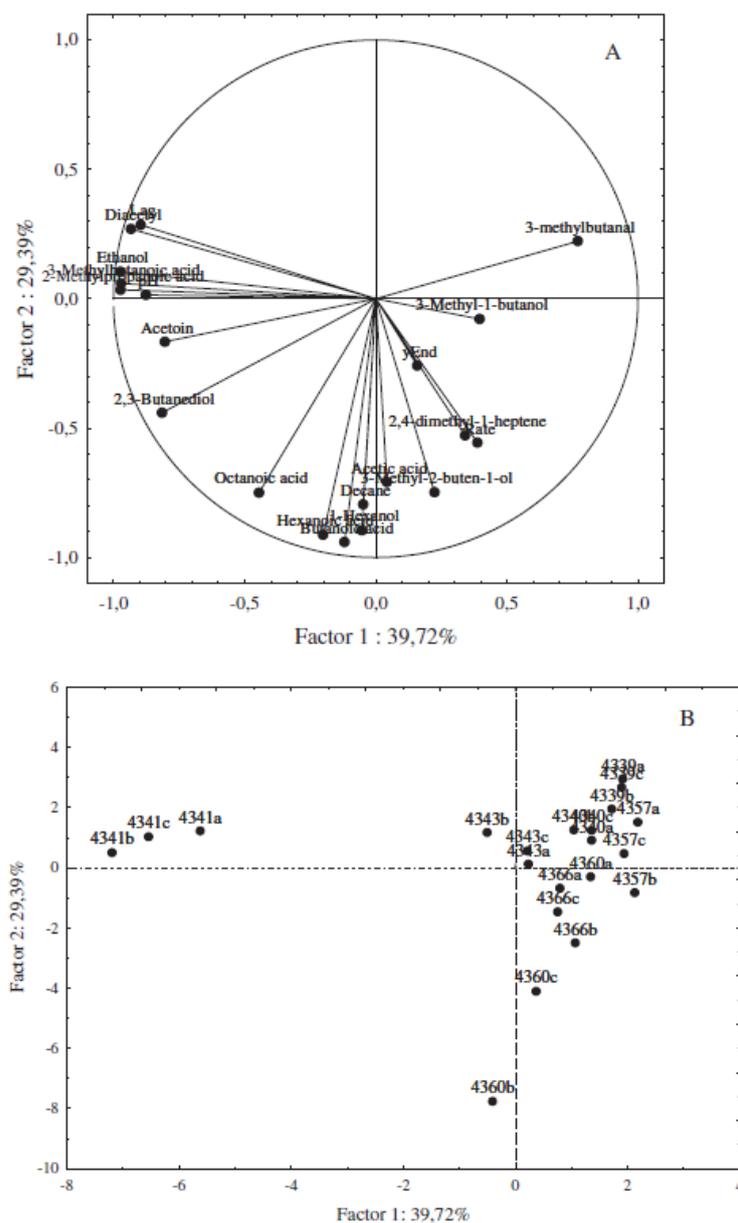
production of 3-methyl-butanoic acid, 2-methyl-propanoic acid, diacetyl, acetoin, 2,3-butanediol and ethanol (Table 4). Diacetyl, acetoin and 2,3-butanediol give, to different extents, an aromatic note related to a pleasant buttery/creamy odour, while 3-methyl-butanoic acid and 2-methyl-propanoic acid give stronger cheese aromatic notes.

From a metabolic point of view, carboxylic acids as 3-methyl-butanoic and 2-methyl-propanoic acid are short branched chain acids deriving from amino acids catabolism, and respectively from leucine and valine, *via* α -keto acids by the transaminase pathway (Liu et al., 2008; Smit and Smit, 2005), involving the production of branched-chain aldehydes. Aldehydes then can be reduced to alcohols by alcohol dehydrogenases (e.g. 3-methylbutanal to 3-methyl-1-butanol) or oxidized to carboxylic acids by an aldehyde dehydrogenase (e.g. 3-methylbutanal to isovaleric acid) (Marilley and Casey, 2004). The production of diacetyl, acetoin and 2-3 butandiol instead, could be linked to the conversion of pyruvate, deriving from lactose or citrate catabolism (Liu, 2003; Smid and Kleerebezem, 2014). As described above, *L. paracasei* 4341 was the slowest to adapt in milk (higher Lag value), but at the same time had a high acidification rate (low Rate value) and low acidification capacity (Yend). Considering that, we can hypothesize that the slow adaptation of this strain to milk could be explained in a difficulty to ferment lactose, to which, the bacteria may overcome with the use of citrate and amino acids. This feature can be of great industrial interest to avoid overlapping acidification with primary starters (Gobbetti et al., 2015).

Acetic acid mainly derives from the heterolactic fermentation of lactose, and its production from *L. paracasei* 4341 strain was below the average value and the pH reached at the end of fermentation was the highest among all strains (pH=4.25, after 5 days). This could be due to the production of 2,3 butandiol which is a neutral compound that decrease acid formation. The relative lower amount of the sour aromatic note related to acetic acid can be considered a positive aspect. The PCA analysis performed taking into account both the volatile profiles, the kinetic parameters and

pH shows the distribution of the strains, cultivated in triplicate, along the first component (39,72%) and the second one (29,39%) (Figure 3). In particular, the strain *L. paracasei* 4341 was well separated from the other strains not only for the acidifying parameters (pH and Lag) but also for all the ketones, as well as the majority of alcohols and acids except for 3-methyl-1-butanol and acetic acid. Interestingly, a high value of Lag is correlated with a high value of diacetyl, supporting our speculation.

Figure 3. Plots of loading (A) and score (B) obtained by PCA analysis of the pH, acidifying kinetic parameters and volatile molecules of the 7 strains cultivated in triplicate (a,b,c) in milk.



Even if not well separated such as *L. paracasei* 4341, the only *L. casei* (putative) of the studied strains, *L. casei* 4339 in triplicate, is positioned in the highest and most right part of the of the plot. This strain was characterized by a high production of 3-methylbutanal and 3-methyl-1-butanol. These two related compounds are known as important aromatic compounds able to impart a fruity/green/nutty note.

L. paracasei 4360 and 4366 showed the highest production of 1-hexanol that is related to a pleasant fruity/flower aromatic note. Furthermore, *L. paracasei* 4360 showed the highest production of acetic, butanoic and hexanoic acids. These last two compounds are short chain fatty acids liberated by lipolysis and are known to be involved in sensory quality of products: due to their strong aroma and low sensory threshold, can lead to an undesired flavor perceived as fermented milk or rancid (Rao et al., 1984). However, these characteristics do not have enough weight to well separate the two strains from the others on the plot. .

2.4 Conclusion

In this study, a novel focused approach was used to isolate and select strains potentially useable as adjunctive aromatic starters in cheese production. Indeed, a spontaneous fermentation at 8°C, and the combining of colony PCR species identification, five day of UHT whole milk fermentation was suitable to isolate and select, directly from the milk, seven strains belonging to *L. casei* group with different ability to grow in milk and to produce aroma compounds. Each strain showed peculiar features that can be exploited in a different way depending on the intended use. Six strains belonged to the *L. paracasei* species and one to putative *L. casei* species. Among all the strain considered, *L. paracasei* 4341 appear the most interesting from the technological point of view both for its acidifying and aromatic features. Particularly, its slow adaptation to milk and its ability to produce considerable amounts of all the appreciate ketones, the majority of alcohols and

acids, suggest its possible use as adjunctive aromatic culture which does not compete with the acidifying starter.

Acknowledgements

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Bancalari, E., Food Research International (2017), <http://dx.doi.org/10.1016/j.foodres.2017.07.066>. Reprinted with license n.4196951089541 from Elsevier

Table 4. Volatile compounds produced by the seven selected strains in UHT whole milk in 5 days at 37°C

Compound	LRI*	Identificat ion method**	Odour Description	Amounts produced by strains (µg/100 mL)***						
				L.c [‡] 4339	L.p [§] 4340	L.p 4341	L.p 4343	L.p 4357	L.p 4360	L.p 4366
2,4-dimethyl-1- heptene	832	MS, RI	No specific aromatic notes	0.47±0.09 ^a	1.1±0.2 ^a	0.5±0.3 ^a	1.0±0.6 ^a	1.0±0.4 ^a	1.2±0.2 ^a	0.9±0.1 ^a
Decane	100 0	MS, RI	No specific aromatic notes	0.04±0.02 ^a	0.02±0.02 ^a	0.05±0.03 ^a	0.03±0.03 ^a	0.03±0.03 ^a	0.08±0.05 ^a	0.2±0.2 ^a
<i>Total hydrocarbons</i>				<i>0.53</i>	<i>1.12</i>	<i>0.55</i>	<i>1.03</i>	<i>1.03</i>	<i>1.28</i>	<i>1.1</i>
3-methylbutanal	820	MS,RI	Fruity, dry, slightly green, chocolate and nut-like	0.98±0.08 ^{bc}	0.81±0.06 ^{bc}	0.29±0.08 ^a	0.95±0.09 ^{bc}	1.1±0.1 ^c	0.60±0.08 ^{ab}	1.0±0.3 ^{bc}
Diacetyl	987	MS, RI, STD	Buttery, cream	0.43±0.09 ^a	0.3±0.1 ^a	2.4±0.5 ^b	0.65±0.03 ^a	0.3±0.1 ^a	0.18±0.5 ^a	0.4±0.1 ^a
Acetoin	129 6	MS, RI, STD	Buttery, cream	1.24±0.06 ^a	16±1 ^c	30±1 ^e	22.1±0.6 ^d	2.7±0.6 ^a	11±2 ^b	16±2 ^c
<i>Total aldehydes/ketones</i>				<i>2.65</i>	<i>17.39</i>	<i>32.45</i>	<i>23.75</i>	<i>4.1</i>	<i>11.38</i>	<i>17.4</i>
3-Methyl-1-butanol	121 0	MS, RI	Whiskey, alcoholic, fruity	1.55±0.05 ^c	0.46±0.9 ^a	0.62±0.05 ^{ab}	0.27±0.04 ^a	1.96±0.9 ^c	0.5±0.02 ^a	1.4±0.4 ^{bc}
3-Methyl-2- buten- 1-ol	132 7	MS, RI	Green, fruity	0.75±0.03 ^{abc}	0.41±0.05 ^a	0.53±0.05 ^{ab}	0.36±0.01 ^a	0.87±0.09 ^{bc}	0.9±0.2 ^{bc}	0.93±0.05 ^c
1-Hexanol	135 7	MS, RI	Floral, fruity	0.052±0.003 ^a	0.146±0.001 ^{ab}	0.14±0.05 ^{ab}	0.116±0.007 _{ab}	0.14±0.05 ^{ab}	0.29±0.06 ^c	0.20±0.06 ^{bc}
2,3-Butanediol	163 2	MS, RI, STD	Fruity, cream, buttery	0.027±0.004 ^a	0.022±0.004 ^a	2.9±0.9 ^c	0.035±0.02 ^a	0.12±0.03 ^a	1.0±0.6 ^{ab}	1.45±0.01 ^b
Ethanol	959	MS, RI	Alcoholic	0.23±0.01 ^a	0.31±0.03 ^a	5.5±0.5 ^b	0.33±0.03 ^a	0.10±0.02 ^a	0.44±0.07 ^a	0.46±0.01 ^a
<i>Total alcohols</i>				<i>2.61</i>	<i>1.35</i>	<i>9.70</i>	<i>1.12</i>	<i>3.19</i>	<i>3.14</i>	<i>4.47</i>
Acetic acid	146 1	MS, RI, STD	Sour	10.3±0.5 ^a	30.3±0.5 ^{cd}	16.9±0.9 ^{ab}	42±3 ^{de}	15±2 ^{ab}	43±9 ^e	22±3 ^{bc}

2-Methylpropanoic acid	1589	MS, RI	Acidic, sour, cheese, buttery, rancid	0.16±0.01 ^a	0.19±0.02 ^a	1.66±0.03 ^c	0.23±0.04 ^{ab}	0.18±0.03 ^a	0.26±0.07 ^{ab}	0.31±0.03 ^b
Butanoic acid	1640	MS, RI, STD	Rancid, cheese, sweat	2.87±0.05 ^a	3.39±0.08 ^a	3.9±0.4 ^a	3.7±0.2 ^a	4.1±0.9 ^a	4.8±0.9 ^a	4.0±0.6 ^a
3-Methylbutanoic acid	1680	MS, RI	Cheese, sweat	0.59±0.06 ^a	0.70±0.01 ^a	35±4 ^b	0.76±0.05 ^a	0.8±0.1 ^a	2.8±0.9 ^a	2.43±0.01 ^a
Hexanoic acid	1835	MS, RI, STD	fatty, cheesy, waxy, fruity	4.8±0.4 ^a	5.2±0.5 ^a	7.1±0.7 ^a	7.0±0.5 ^a	7.3±0.9 ^a	7.6±0.9 ^a	7.6±0.7 ^a
Octanoic acid	2100	MS, RI	Sweat, cheesy	1.1±0.2 ^a	1.39±0.04 ^{ab}	2.41±0.08 ^c	2.3±0.3 ^{bc}	1.73±0.04 ^{abc}	1.9±0.7 ^{abc}	2.5±0.3 ^c
<i>Total acids</i>				<i>19.86</i>	<i>41.18</i>	<i>67.44</i>	<i>55.65</i>	<i>29.39</i>	<i>60.1</i>	<i>38.88</i>
<i>Total</i>				<i>25.63</i>	<i>61.03</i>	<i>110.13</i>	<i>81.58</i>	<i>37.81</i>	<i>75.89</i>	<i>62.27</i>

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Chapter 3

Rheological characteristics of milk fermented by three eps-producing lactic acid bacteria

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3.1 Introduction

Lactic acid bacteria (LAB) have traditionally been associated with food fermentation due to their long and safe history of application and consumption in the production of fermented food and beverages.

All the desirable attributes that LAB are able to transmit to specific fermented foods are due to specific traits that they possess, above all, the production of lactic acid from the carbon source as well as the ability to form other compounds such as vitamins, bioactive peptides, aroma compounds, and exopolysaccharides (EPS) (Torino et al., 2015). EPS are biomolecules that exhibit heterogeneous composition, structure and a broad range of physicochemical properties and application (Malang et al., 2015; Galle and Arendt, 2014). They are long chain molecules consisting of branched, repeating units of sugars, or sugars derivatives, and can be classified according to their chemical composition and biosynthesis mechanism into two main groups: Homopolysaccharides (HoPS) and Heteropolysaccharides (HePS) (Torino et al., 2015). HoPS contain one neutral monosaccharide type either glucose (glucans), fructose (fructans), or galactose (polygalactan), but they could differ in terms of glycosidic bonds, branching, chain length, molecular weight and polymer structure (Oleksy and Klewicka, 2016). In contrast to HoPS, HePS are complex polymers composed of a backbone of repeating subunits, branched or unbranched, that consist of three to eight monosaccharides, derivatives of monosaccharides or substituted monosaccharides. HePS usually contain D-glucose, D-galactose, and L-rhamnose although in different ratios. To a lesser extent fructose and fucose and other residues such as N-acetylated monosaccharides (N-acetyl-glucosamine and N-acetyl-galactosamine), as well as organic and inorganic (glucuronic acid, acetyl groups, glycerol, phosphate, etc.) can also be present (De Vuyst and Degeest, 1999; Degeest et al., 2001). Being macromolecules, the molecular weight of EPS is high, with HePS ranging from 10^4 to 10^6 Da, which is generally lower than the average molecular

mass of HoPS which ranges up to approximately 10^7 Da (Sanlibaba and Çakmak, 2016; Oleksy and Klewicka, 2016).

The biological role of EPS production in LAB has not yet been clearly established. On the one hand, it was suggested that their role was as a nutrient reserve, but on the other hand, most of the EPS-forming bacteria lack the EPS degrading enzyme (Galle and Arendt, 2014). However, in their natural environment, EPS were found to protect the microbial cell from environmental stresses, as well as playing a role in biofilm formation, cellular recognition and quorum sensing control, and surface adhesion (Galle and Arendt, 2014; Dertli et al., 2015).

Apart from these ecological functions, EPS from LAB have technological significance when they are employed in the production of fermented food or in the development of functional food products (Ruas-Madiedo et al., 2002; Zannini et al., 2015; Sanlibaba and Çakmak, 2016) because of their thickening, emulsifying, gelling and stabilizing abilities (Li et al., 2012). The increased demand for natural-origin polymers for various industrial applications in recent years has led to a renewed interest in EPS production by microorganisms. Those bacterial polymers have emerged as new, technologically effective biomaterials, which are gradually proving to be economically comparable with natural gums produced by marine algae or other plants (Kumar et al., 2007; Mende et al., 2012). Furthermore, the consumer pays increasingly more attention to the relationship between food and health and the use of food additives is regarded as unnatural and unsafe (Ruas-Madiedo et al., 2002; Galle and Arendt, 2014). Moreover, the increasing demand for low-fat and low calorie dairy products that are now expected to be made without additives, but should exhibit a creamy and smooth texture (Mende et al., 2012). For these reasons, the *in situ* production of LAB EPS contributes to the emerging policy of food industries to reduce the substitute additives with natural molecules so as to have a “clean label” (Varela and Fiszman, 2013).

The natural variety of EPS producing LAB are reflected in a wide diversity and variability in EPS production in terms of quantity, chemical composition, molecular size, charge, presence of side chains and the rigidity of molecules, thus providing different useful functional properties when used in food systems. Application examples of EPS-producing cultures have been recorded for the production of fermented milks and beverages where they are applied in order to reduce the amount of added milk solids, to improve the viscosity, texture, stability and mouthfeel of the final product as well as to avoid or reduce syneresis during fermentation or upon storage (Torino et al.,2015). It has also been reported that EPS may act, especially in fermented milk products, as texturizers and stabilizers, firstly increasing the viscosity and the mouthfeel of the final product and secondly by binding hydration water and interacting with other milk constituents to strengthen the rigidity of the casein network, therefore decreasing syneresis (Leroy and De Vuyst, 2004).

Even if the *in-situ* production of these biopolymers by LAB has been extensively studied during the last decade, there are still many unknown facts when working with the EPS-producing LAB strains. In our opinion, one of the major difficulties in using *in situ* EPS production is their strain-dependence and their strongly affected by microbial culture condition (Zannini et al.,2015; Sanlibaba and Çakmak, 2016). A deeper understanding of the cultural conditions allowing EPS production is a crucial step in order to have a reproducible output, a fundamental requisite for industrial use, requiring controlled processes. With regards to this context, this research wants to take a step forward. In this study we proposed an approach to evaluate an *in situ* production of EPS by three LAB strains: two belonging to same species *Lactobacillus delbrueckii* subsp. *bulgaricus* commonly used as starter in fermented milk production and one, *Weissella*, typically isolated from sourdough but able to develop in milk and produce EPS. The different modification of the rheological characteristics of the milk in which the strains have developed and have produced EPS

suggests their potential different uses as bio thickeners to produce “clean label” fermented dairy products.

EPS produced by the three strains have been characterized in terms of relative composition in monosaccharides by GC-MS analysis (Table 1). All the three strains were able to produce HePS, which differ either in the monosaccharides composition of the repeating unit, or in the percentage of their presence (Table 1). EPS from *Lb. delbrueckii* subsp. *bulgaricus* 2214 and 147 differed in the presence of the main monosaccharide which was glucose for the first strain and galactose for the latter. Moreover, EPS from *Lb. delbrueckii* subsp. *bulgaricus* 2214 was principally (90%) composed by glucose and the remaining 10% consisted of rhamnose and mannose. EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* 147 was composed of 5 different monosaccharides, present in different ratios. In this case, 90% of the EPS was comprised of an equivalent amount of two principal monosaccharides: glucose and galactose (Table 1). The composition of the EPS produced by *Weissella* 4451 was more similar to the EPS from *Lb. delbrueckii* subsp. *bulgaricus* 147. Our results are in agreement with the structural diversity of EPS produced by LAB previously observed (Zeidan et al.,2017), confirming that galactose and glucose, and to a lesser extent mannose and rhamnose, are the most frequently occurring sugars in repeating units of exocellular polysaccharides in lactobacilli.

3.2 Materials and methods

3.2.1 Strains

Three strains (*Lactobacillus delbrueckii* subsp. *bulgaricus* 2214 EPS+, *Weissella* sp.4451 EPS+, and *Lactococcus lactis* 220 EPS-) belonging to the collection of Food Microbiology Laboratory of the Food Science and Drug Department (University of Parma) and one (*Lb. delbrueckii* subsp. *bulgaricus* 147 EPS+), kindly provided from Sacco Srl (Cadorago, Italy) were used.

The strains, were maintained at -80°C as frozen stocks cultures in MRS broth for *Lactobacillus* and *Weissella* (Oxoid, Ltd., Basingstoke, United Kingdom), and M17 (Oxoid, Ltd.) broth for *Lactococcus* containing 20% (v/v) glycerol. They were recovered by anaerobic incubation in the same media, by two overnight sub-culturing (2%v/v) at optimal temperatures of 42°C for *Lactobacillus* and 30°C for *Lactococcus* and *Weissella*.

3.2.2 EPS characterization by gas chromatography-mass spectrometry

The strains were cultivated in Semi-Defined Medium (SDM), useful to provide minimal interference with the assays used to characterize EPS (Kimmel and Roberts, 1998). SDM was sterilized by heating for 15 min at 121°C and the lactose (Merck, Darmstadt, Germany) aqueous solution (60g/L), that was used as carbohydrate source, was autoclaved separately (15 min at 121°C) and aseptically added to the medium. The inoculated (2% v/v) broths were incubated at non-optimal temperature of 37°C for *Lactobacillus* and *Weissella sp.* (Nguyena et al., 2014), and 30°C for *Lc. lactis*. Total EPS from the SDM medium were extracted as previously reported (Rimada and Abraham, 2003). The purity of the EPS from protein and nucleic acid was assessed by UV-visible spectrophotometer (Jasco V-530), at 260-280 nm (Liu et al., 2016).

EPS recovered were hydrolyzed with 3 ml 2 M trifluoroacetic acid under nitrogen for 60 min at 121°C . To the hydrolyzed mixture, 1 ml phenyl-beta-D-glucopyranoside (500 ppm) was added as internal standard. The samples were clarified by syringe filtration on nylon filters ($40\ \mu\text{m}$) and the filtrate evaporated to dryness with nitrogen. Sugars were silylated for 60 min at 60°C adding $600\ \mu\text{l}$ N-N-dimethylformamide and $200\ \mu\text{l}$ N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS). The gas chromatography-mass spectrometry (GC/MS) analysis was carried out using apolar capillary column (HP-5MS, Hewlett.Packard) in the conditions previously reported (Müller-Maatsch et al., 2016).

GC-MS analysis were performed in duplicate. Identification was performed by comparison with retention times and mass spectra of sugars standards (mannose, xylose, ribose, glucose, galactose, rhamnose, arabinose, fucose). Quantification was performed by means of internal standard and results are finally expressed as relative percentages.

3.2.3 Milk fermentation

Three recovered EPS+ strains were used to perform two sub-culturing (2% v/v) at non-optimal temperature (37°C) in skim milk powder (SSM) (Oxoid Ltd.) reconstituted to 9% (w/v) and sterilized at 110°C for 15 min (SSM). As negative control one EPS- strain was used to perform two sub-culturing (2% v/v) at 30°C in SSM. 2% of the last overnight sub-cultures were used for batch fermentation, that was performed in a bioreactor (working volume 0.5 L; Applikon Biotechnology, Schiedam, The Netherlands), which was sterilized at 121°C for 15 min.

The fermenter was equipped with software BioXpert Lite (Applikon Biotechnology®) for system control and data acquisition. The temperature was constantly recorded by Pt-100 sensor (Applikon Biotechnology®), and pH by means of Applisensor pH gel sensor (Applikon Biotechnology®).

The fermentation was carried out at non-optimal temperature (37°C) for *Lactobacillus* and *Weissella*, and 30°C for *Lc. lactis* and stopped cooling to 4°C when the pH reached the pH set value of 4.5.

3.2.4 Viscosity measurement of fermented milk

At the end of fermentation, the coagulum was broken by means of a rod stirrer (DLH VELP Scientifica®) at 210 rpm for 5 minutes, and then it was maintained at 4°C for 24 h.

Afterwards the fermented milk was equilibrated at 25°C for 90 min and again stirred at 80 rpm for 5 min in order to reduce possible structural differences among samples caused by the fermentation procedures. After an equilibration time that helps to reorganize the matrix structure

solely due to the chemical characteristics of the sample (Purwandari et al., 2007), the viscosity was measured by means of a Brookfield® DV-I Prime rotational viscometer (Middleboro, Massachusetts, USA) equipped with a SC4-18 spindle. The temperature was maintained at $25^{\circ}\text{C} \pm 0.5$ by connecting the Small Sample Adapter chamber (Middleboro, Massachusetts, USA) to a thermostatic water bath. The viscometer was connected to a computer for the data acquisition at 1 sec intervals in an ASCII table. Apparent viscosity ($\eta = \sigma/\dot{\gamma}$) was measured between the range of shear rate from 0.792 and 7.92 s^{-1} . The flow behavior of the samples was described by the Ostwald de Waele model (Power Law): $\sigma = k \dot{\gamma}^n$, where σ is the shear stress (Pa), k is the consistency index ($\text{Pa}\cdot\text{s}^n$), $\dot{\gamma}$ is the shear rate (s^{-1}) and n is the flow behaviour index.

The viscosity of the fermented milk samples was also measured by means of a flow cup (brass Ford Cup Sacco Srl (Cadorago, Italy), capacity of 100 ml, with an outflow opening diameter of 3 mm, according to the standard ISO 2431 (2011) with the following slight modification. Due to the high viscosities showed by the samples, it was decided to express Ford Cup viscosity as g of sample eluted in 3 minutes, instead of time necessary for the elution of the total volume (100 ml). For the measurement, the Ford Cup was filled with the fermented milk at 25°C .

3.2.5 Determination of water holding capacity of fermented milk

Water holding capacity (WHC) is defined as the ability of the fermented milk containing EPS to hold water. WHC of the fermented milks at pH 4.5, were determined by the centrifugation method described by Li and colleagues (Li et al., 2014). Twenty grams of each fermented milk were centrifuged at 2600 rpm for 15 min. The supernatants were collected, weighed, and WHC was calculated according to the following equation: $\text{WHC} (\%) = 1 - (W1/W2) * 100$, where: $W1$ = weight of supernatant after centrifugation (g) and $W2$ = weight of the fermented milk before centrifugation (g).

3.2.6 Exopolysaccharides quantification in fermented milk

The procedure for isolation and purification of free EPS previously reported by Mende (Mende et al.,2012) and Rimada and Abraham, (Rimada and Abraham, 2003) was used. After adding 0.7 mL 80% (w/v) trichloroacetic acid (TCA) and heating to 90°C for 15 min, samples were cooled in ice water and centrifuged (2000 rpm, 20 min, 4°C) to remove cells and protein. After neutralization of the supernatant with NaOH, the EPS were purified following the procedure reported by Rimada and Abraham (Rimada & Abraham, 2003) and the quantification of the polymers was determined as polymer dry mass (PDM) (Van Geel-Schutten et al.,1998). Samples were centrifuged (20 min at 2600 rpm), and the pellets were dried at 55°C. Dried EPS amounts were weighted using an Acculab Precision Analytical Balances with 4 decimal places.

3.2.7 Statistical analysis

For each parameter considered, one-way ANOVA and Tukey-HSD post hoc tests ($\alpha = 0.05$) were performed to detect significant differences among the different analysis performed for each strain. Data of every parameter considered were obtained from two independent fermentation processes and each sample was analyzed in triplicate. The degree of correlation for the parameters measured was checked by means of Pearson correlation coefficients (r); the correlation was considered significant at an $\alpha = 0.05$.

3.3 Results and discussion

3.3.1 EPS Characterization

EPS produced by the three strains have been characterized in terms of relative composition in monosaccharides by GC-MS analysis (Table 1). All the three strains were able to produce HePS,

which differ either for monosaccharides composition of the repeating unit, or for the percentage of their presence (Table 1).

Table1. Monosaccharides composition of EPS, characterized by GC-MS analysis, reported as relative percentage.

Monosaccharide	Relative percentage of composition and SD		
	<i>Lb. delbrueckii</i> subsp. <i>bulgaricus</i> 147	<i>Lb. delbrueckii</i> subsp. <i>bulgaricus</i> 2214	<i>Weissella</i> 4451
Rhamnose	2.8 ± 0.3	9.1 ± 0.8	12.4 ± 1.1
Ribose	0.8 ± 0,1	nd	0.5 ± 0.5
Mannose	6.0 ± 0.5	0.9 ± 0.1	12.6 ± 1.1
Galactose	48.2 ± 4.3	nd	17.5 ± 1.6
Glucose	42.2 ± 3.8	90.0 ± 8.0	57.0 ± 5.1

Abbreviations: nd, not detected, SD, standard deviation

EPS from *Lb. delbrueckii* subsp. *bulgaricus* 2214 and 147 differed for the presence of the main monosaccharide which was glucose for the first strain and galactose for the latter. Moreover, EPS from *Lb. delbrueckii* subsp. *bulgaricus* 2214 was principally (90%) composed by glucose and the remaining 10% consisted of rhamnose and mannose. EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* 147 was composed by 5 different monosaccharides, present in different ratio. In this case, the 90% of the EPS was constituted by an equivalent amount of two principal monosaccharides: glucose and galactose (Table 1). The composition of the EPS produced by *Weissella* 4451 was more similar to the EPS from *Lb. delbrueckii* subsp. *bulgaricus* 147. Our results are in agreement with the structural diversity of EPS produced by LAB previously observed (Zeidan et al., 2017), confirming that galactose and glucose, and to a lesser extent mannose and

rhamnose, are the most frequently sugars occurring in repeating units of exocellular polysaccharides in lactobacilli.

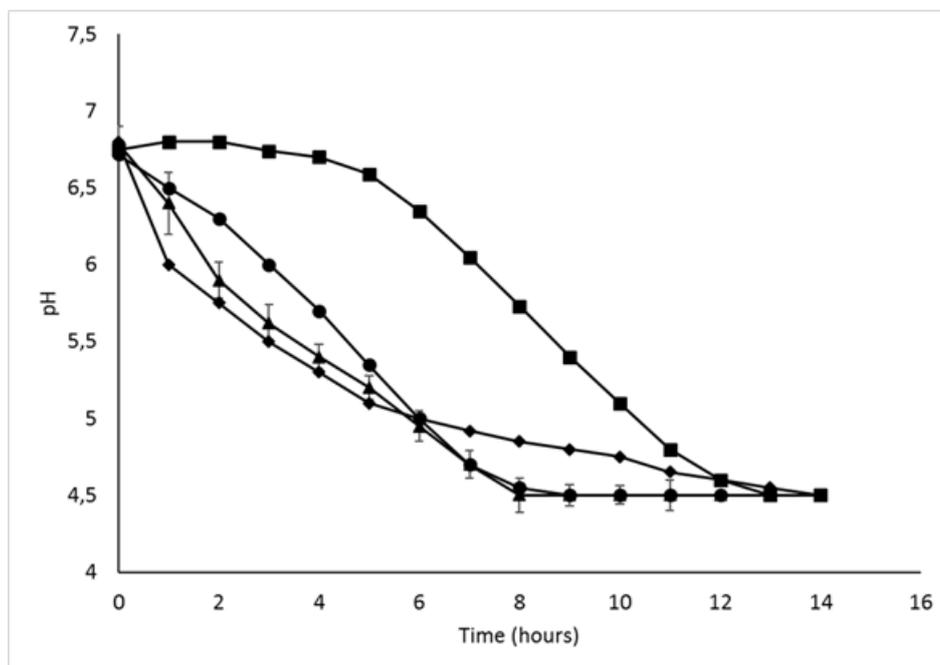
3.3.2 Milk fermentation

To evaluate the modification of the rheological characteristics of the milk in which the different EPS have been produced, the three strains EPS+ were cultivated in SSM at the non-optimal temperature of 37°C (Nguyena et al., 2014). As a negative control, the EPS- strain kept at 30°C was considered. In these conditions, the four strains showed diverse acidification and growth rate, reaching the fixed pH value of 4.5 at different rates (Fig. 1).

The acidification rate was variable among all the strains, in particular, the three strains EPS+ showed a higher acidification rate compared to the strain EPS- *Lc. lactis* 220 (Fig. 1). *Weissella* 4451 reached the pre-determined pH value in approximately 8 hours, proving to be the strain with the highest acidification rate.

On the other hand, the two strains belonging to the same species, *Lb. delbrueckii* subsp. *bulgaricus* 2214 and 147, reached the pre-determined pH in 9 hours and 13 hours respectively, showing very different acidification rates.

Figure 1. Batch fermentation profile of (●) *Lb. delbrueckii* subsp. *bulgaricus* 2214, (◆) *Lb. delbrueckii* subsp. *bulgaricus* 147, (▲) *Weissella* 4451, (■) *Lc. lactis* 220, in SSM at non-optimal temperature.



Interestingly, *Lb. delbrueckii* subsp. *bulgaricus* intraspecific diversity was observed in a previous research where 20 different strains showed the different ability to coagulate milk and showed a greater heterogeneous behavior in all the acidifying parameters studied (Bancalari et al., 2016).

Once the fermentation was stopped, it was possible to observe differences in the aspect of the coagulated milk, formed by the EPS+ strains. The strains *Lb. delbrueckii* subsp. *bulgaricus*2214 and *Weissella*4451 produced a more compact and uniform clot, compared to the one obtained from the fermentation with strain EPS+ *Lb. delbrueckii* subsp. *bulgaricus*147, which appeared grainy and more similar to the clot obtained from the fermentation carried out with the strain EPS- (data not shown).

3.3.3 Exopolysaccharides production and rheological characteristics in fermented milk

It is known that the amount and effect of EPS synthesized *in situ* can vary considerably, and depends on the strain, substrate and fermentation conditions (Mende et al.,2013). In our case, at the end of SSM fermentation, when pH 4.5 was reached, significantly different amounts of EPS, varying from 1,88 g/L for the strain *Lb. delbrueckii* subsp. *bulgaricus* 2214 to 0.96 g/L for *Lb. delbrueckii* subsp. *bulgaricus* 147 were found (Table 2). Due to the known limit of the EPS extraction procedure, a low amount of polymers dry mass was also found for the EPS- strain probably due to the lactose moieties (Rühmann et al.,2015). It was very interesting to note that *Weissella* 4451, which was isolated from sourdough, was able to produce a good quantity of EPS in milk, similar to the best producer *Lb. delbrueckii* subsp. *bulgaricus* 2214.

It is known that EPS production is generally accompanied by an increase in the apparent viscosity of the medium (Mende et al.,2013). The rheological data (shear rate vs shear stress) obtained from this study were well fitted to the power law model with high determination coefficients ($R^2 = 0.94 \sim 0.99$).

All the fermented milks showed a shear-thinning behavior (Table 2). Values of flow behavior indexes (n) varied from 0.353 ± 0.112 of *Lb. delbrueckii* subsp. *bulgaricus* 147, which had the most pseudoplastic behavior, to 0.578 ± 0.071 of *Weissella* 4451. Also the apparent viscosity ($\eta_{3.96}$) showed significant differences amongst all samples. These two parameters appear to be correlated to the different amount of EPS although it has been observed that the different composition of the EPS could influence the ability to interact with milk proteins and modify viscosity independently from the amount (Purohit et al.,2009).

Table 2. Exopolysaccharides production (PDM), viscosity (measured by mean of Ford Cup and Viscometer) and water holding capacity in milk fermented by three EPS+ and one EPS- strains. Data were obtained from two independent fermentation processes, and each sample was analyzed in triplicate.

Strains	EPS Production	Viscosity			Water Holding Capacity	
		Ford Cup	Viscometer			
	PDM g/L	g/3min	n (-)	K(Pa*s ⁿ)	$\eta_{3.96}$ (Pa*s)	WHC
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> 147 (EPS+)	0.96 ^c	3.66 ^b	0.353 ^c	0.863 ^b	0.349 ^c	37.72% ^c
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> 2214 (EPS+)	1.88 ^a	1.03 ^a	0.553 ^{a,b}	1.182 ^a	0.667 ^a	51.88% ^a
<i>Weissella</i> sp. 4451 (EPS+)	1.58 ^b	1.73 ^a	0.578 ^a	0.844 ^b	0.500 ^b	41.59% ^b
<i>Lactococcus lactis</i> 220 (EPS-)	0.21 ^d	9.73 ^c	0.435 ^{b,c}	0.492 ^c	0.222 ^d	35.28% ^d

Values (mean of the triplicate)sd followed by different letters within the same column are significantly different (Tukey's HSD, $p < 0.05$).

Abbreviations: PDM, polymer dry mass; n (-), flow behaviour index; K(Pa*sⁿ), consistency index ; $\eta_{3.96}$ (Pa*s), apparent viscosity; WHC, water holding capacity

K coefficient indexes were positively correlated with the viscosity values for *Lb. delbrueckii* subsp. *bulgaricus* 2214 and *Lc. lactis* 220 but not for *Lb. delbrueckii* subsp. *bulgaricus* 147 and *Weissella* 4451 that didn't show significant differences in the K index although it had different viscosity.

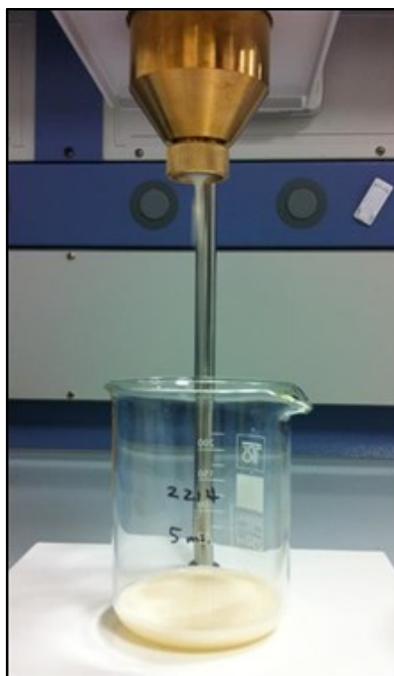
The best producers *Lb. delbrueckii* subsp. *bulgaricus* 2214 and *Weissella* 4451 determined significantly higher values of $\eta_{3.96}$, when compared with *Lb. delbrueckii* subsp. *bulgaricus* 147 and the EPS- *Lc. lactis* 220. In particular, *Lb. delbrueckii* subsp. *bulgaricus* 2214 showed the highest values of consistency index and apparent viscosity, indicating a thick and firm structure (Table 2).

As expected, the EPS- *Lc. lactis* 220 produced the fermented milk with the lowest apparent viscosity and consistency index (Hassan et al.,2003).

The different amounts of produced EPS was directly correlated ($r = 0.93$, $p < 0.001$) with the measured apparent viscosity suggesting that the strains producing higher amount of EPS might contribute to the higher viscosity of fermented milk. In particular, milk fermented by *Lb. delbrueckii* subsp. *bulgaricus* 2214, showed a viscosity value significantly higher than that of the other strains ($p < 0.05$). Positive correlation is in agreement with Guzel-Seydim, Sezgin and Seydim, (2005), which reports that EPS filaments may attached mucous bacteria to the protein matrix and thus cause more viscous-like behavior.

An alternative method to measure viscosity is represented by the use of Ford Cup which is an empirical method commonly used with varnishes and paints. The procedure refers to ISO 2431, (2011) "Paints and varnishes — Determination of flow time by use of flow cups" which identifies Ford Cup as a method for determining the flow time of paints, varnishes and related products which can be used to check consistency. In this paper however, the flow cup was used as an easy and alternative method to measure the viscosity of fermented milks, by weighing the milk flowing through an orifice of 3 mm diameter in 3 minutes at 25°C (Fig. 2). The milk fermented with EPS- *Lc. lactis* 220 showed the highest flow rate, therefore being the less viscous sample. For the other strains tested, a lesser quantity of fermented milk was weighed after passing through the Ford Cup, with the milk fermented by *Lb. delbrueckii* subsp. *bulgaricus* 2214 resulting as the most viscous (Table 2).

Figure 2. Ford Cup used to measure the viscosity of fermented milk by weighing the milk flowing through an orifice of 3 mm diameter.



Interestingly, even if Ford Cup analysis was not able to significantly discriminate *Lb. delbrueckii* subsp. *bulgaricus* 2214 from *Weissella* 4451, the other data were in sufficient agreement ($r = 0.85$, $p < 0.001$) with apparent viscosity measured using the viscometer, allowing the discrimination between the highest producers and the lowest ones (Table 2).

3.3.4 Water Holding Capacity measurement

In addition to rheological characteristics, EPS can modify the textural and the physical properties of fermented milk products improving the mouthfeel and the consistency of the products by interacting with the free water in the gel-like structure and delaying or limiting syneresis during shelf life (Han et al., 2016). For these reasons, Water Holding Capacity was also measured.

The results of the WHC measurements (Table 2) showed different behaviors between strains EPS+ and EPS-; in particular, the WHC of the milk fermented by the EPS- strain was significantly lower compared to all the other EPS+ fermented milk samples. Above all, the strain *Lb. delbrueckii* subsp. *bulgaricus* 2214, which was the best EPS producer, showed the significantly highest WHC value, which is in agreement with the highest value of viscosity (Han X. et al., 2016), as a more viscous matrix improves the stability against water syneresis, and it could also be in relation with the higher EPS amount (Table 2).

It is known that the impact of EPS-producing cultures on physical properties may be in relation with milk properties and EPS characteristics and/or amounts (Ruas-Madiedo et al., 2002). Differences in WHC observed in this study are correlated with the quantity of EPS produced by the different strains ($r = 0.87$, $p < 0.001$) and consequently to their viscosity. Moreover, the large differences measured in terms of type and relative amount of monosaccharides in the EPS can also contribute to the explanation of these differences.

3.4 Conclusions

The approach used in this study, to evaluate the *in situ* production of EPS by three LAB strains, allowed us to observe that *Lb. delbrueckii* subsp. *bulgaricus* 147 and 2214 strains were able to produce different amounts of two EPS with different monomeric compositions. They were differently able to modify the rheological characteristics of the milk: the higher the amount of EPS, the higher the flow behavior index, the shear rate and the water holding capacity of the fermented milk.

Weissella 4451, isolated from sourdough, was able to produce EPS in milk with a similar monomeric composition to the EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* 147.

The different capacity of these three LAB strains to modulate the rheological characteristics of milk during fermentation, suggests their potential uses as bio thickeners to produce “clean label” fermented dairy products.

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Chapter 4

Impedance microbiology: a step forward in lactic acid bacteria exopolysaccharides detection in fermented milk

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4.1 Introduction

Lactic acid bacteria (LAB) have been used around the world to improve the preservation, nutritional value and sensorial characteristics of a variety of fermented foods. Due to their long history of safe use in human consumption, some LAB strains have the Qualified Presumption of Safety (QPS) or the Generally Recognized As Safe (GRAS) status (EFSA, 2010).

In addition to their main feature in food fermentation processing, which is lactic acid production from the carbon sources present in the matrix where they grow, several LAB strains are also able to produce other metabolites such as aroma compounds and exopolysaccharides (EPS) (Leroy and DeVuyst, 2004). As the majority of bacteria, LAB can synthesize exocellular polymers, that includes both capsular polysaccharides (CPS), covalently bound to the cell surface, and EPS, which may form a loosely bound layer that can also be secreted into the environment (Torino et al., 2015). EPS are long-chain polysaccharides consisting of branched, repeating units of sugars or sugar derivatives. EPS from LAB are highly diverse in structure and can be classified according to different criteria. The most frequently used EPS classification is based on their monomer composition. On this basis EPS are split into two major groups: homopolysaccharides (HoPS) and heteropolysaccharides (HePS). The HoPS are repeating units of a single neutral monosaccharide, which can be either glucose (glucans), fructose (fructans) or galactose (polygalactans). In contrast, HePS are complex polymers composed of a backbone of repeating subunits, branched or unbranched that consist of three to eight monosaccharides, derivatives of monosaccharides or substituted monosaccharides. HePS usually contain D-glucose, D-galactose, and L-rhamnose although in different ratios (De Vuyst and Degeest, 1999).

The role of EPS in their natural environment is complex and still unclear. They seem to play a role in cellular recognition and quorum-sensing control, but also in the protection of microbial cell integrity against hostile environments, in surface adhesion and biofilm formation (Dertli et al., 2015). In addition, it has been hypothesized that EPS may represent an extracellular energy/carbon

reserve. However, this role is questionable since most of EPS-producing species lack the genes involved in their own EPS degradation, (Torino et al., 2015; Zannini et al., 2015).

From the perspective of the food industry, EPS-producing bacteria is attracting a lot of interest due to the simplified regulatory hurdles to its application in food products (Zannini et al., 2015). In this respect, efforts have been made to use LAB as microbial factories for the production of industrially safe metabolites (Boguta et al., 2014; Torino et al., 2015). The EPS-producing cultures could totally or partly replace supplementing plant- or seaweed-derived hydrocolloids or milk solids that are currently used in the food industry to improve viscosity, texture, stability and mouthfeel of final products. Moreover, EPS-producing strains may be helpful in avoiding unwanted syneresis (whey separation) during fermentation or upon storage of fermented milk products (Degeest et al., 2001; Galle and Arendt, 2014). Recently, the increasing demand for natural polymers for industrial application has led to a renewed interest in EPS production by LAB, especially for the *in situ* production (Zannini et al., 2015).

The *in situ* EPS production can be evaluated using direct analytical methods or by mean of indirect measurement of the change in viscosity of the environment where they are produced. The visual inspection of LAB bacterial colonies on agar plates is probably the easiest and most widely used method, although it is only indicative and unable to detect the LAB strains that produce low amounts of EPS. Recently, the increasing knowledge of the gene encoding for the production and and regulation of EPS synthesis has led to the development of screening methods at the molecular level (Van der Meulen et al., 2007). LAB EPS production, from genes to industrial applications, has been recently deepening reviewed by Zeidan and colleagues (Zeidan et al., 2017).

Differently from that approach, with this work we hypothesized to use impedance microbiology to directly evaluate the EPS production in milk.

Impedance, applied to microbiology, can be defined as the resistance in flow of an alternating electrical current that passes through a conducting microbial growth medium (Lanzanova et al., 1993, Mucchetti et al., 1994). The growth of microorganisms increases the conductivity of the

medium by converting uncharged or weakly-charged substances present in the culture medium, into highly charged substances (López Rodríguez et al., 2014). This cheap and non-destructive technique works by measuring the impedance change caused by modifications in the medium resistance and in the interface reactance, produced by the growth and metabolism of microorganisms (López Rodríguez et al., 2014).

Thus, measuring the electric signal that quantify the movement of ions between two electrodes, impedance microbiology can be used to evaluate growth and the potential acidifying performances of LAB, (Noble et al., 1999). Plotting of the continuous measurement of the change of electric signal in the growth medium during LAB development, graphically results in an impedance curve (Rediers et al., 2012). Recently, a new method to interpret the significance of impedance curve has been proposed (Bancalari et al., 2016).

This method suggested the use of Three objective parameters, that can be obtained translating the impedance curve by fitting the data 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>). This conversion into three parameters, was possible only if the original and fitted curves overlap (Bancalari et al., 2016). Applying this method to several LAB strains, we observed that the two curves not always overlapped. Intriguingly, this happened when the EPS+ strains were cultivated.

It was therefore hypothesized that the differences in the curves could be due to the presence of EPS synthesized during growth. With the aim to prove this hypothesis, the ability of the EPS+ LAB strains to modify the impedometric curve was firstly investigated in the MRS broth and, subsequently, in milk. In particular, one EPS+ strain, *Lactobacillus delbrueckii* subsp. *bulgaricus* 2214, was cultivated in milk and the shape of the obtained impedometric curve was considered to reveal EPS production and to further understand this phenomenon. Overall, this work aimed at proposing impedance microbiology as an alternative screening approach suitable to evaluate the EPS production by LAB in milk. Since EPS in milk act as texturizing and stabilizing agents by

improving the viscosity and the mouthfeel of the final product and decreasing syneresis, the proposed method would represent a useful tool in biotechnology industries to achieve information.

4.2 Materials and methods

4.2.1 Strains and growth conditions

Twenty-three EPS+ strains: *Streptococcus thermophilus* (8 strains), *Lactobacillus delbrueckii* subsp. *bulgaricus* (3), *Lactobacillus helveticus* (2), *Lactobacillus casei* (1), *Lactobacillus paracasei* (4), *Weissella* (2), *Leuconostoc citreum* (2), *Lactobacillus plantarum* (1), and 1 EPS- strain, *Streptococcus thermophilus*, all belonging to the collection of the Laboratory of Food Microbiology of the Department of Food and Drugs of the University of Parma, were used (Table 2). The strains were previously selected according to their EPS-producing abilities on sucrose-supplemented MRS agar medium with 40 g/L of sucrose instead of glucose (called MRS-sucrose, MRSS) (Bounaix et al., 2009) (data not shown).

Strains, maintained at -80°C as frozen stocks cultures in MRS (Oxoid, Ltd., Basingstoke, United Kingdom) broth containing 20% (v/v) glycerol, were recovered in MRS broth by two overnight sub-culturing (2% v/v) at 42°C for *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus*, and 30° for the other strains. Then, two other sub-culturing (2% v/v) in MRSS or in skim milk powder (Oxoid Ltd.), reconstituted to 10% (w/v) and sterilized at 110°C for 30 min (SSM), were performed for each strain at 37°C before impedometric analysis.

4.2.2 Impedance measurement

The recovered strains were inoculated (2% v/v) into previously sterilized measuring Bac Trac cells filled with 6ml of MRSS broth or SSM and analyzed at 37°C .

A Bac Trac 4300[®] Microbiological Analyzer (Sylab, Austria) system, consisting of two incubators allowing simultaneous setting of four different temperatures, was used to evaluate the impedometric curves obtained by the EPS+ strains. The BacTrac4300[®] system enables a separate registration of

two specific impedance values, the *E*-value (E%) which is referred to as the impedance relative change at the electrode surface, and the *M*-Value (M%) which is the relative change in conductivity in medium (Bancalari et al., 2016). For the experiments carried out in the present study, the E% was recorded every 10 min for 55h.

All the analyses were carried out in triplicate. One negative sample, consisting of non-inoculated MRS and SSM, was also incubated for each temperature tested.

4.2.3 Measure of pH and Exopolysaccharides quantification of *Lactobacillus delbrueckii* subsp. *bulgaricus* 2214 in SSM

The EPS+ *Lactobacillus delbrueckii* subsp. *bulgaricus* 2214 (*L. bulgaricus* 2214) was used as a model strain to investigate whether the fall in the impedometric curves was due to the production of EPS. Sampling (Figure 3) at the beginning of the measurement (T0) and after 8h (T1), 13h (T2) and 55h of incubation (T3) were considered as relevant to this assessment.

The final pH of the SSM inoculated with *L. bulgaricus* 2214 was directly measured inside the measuring cells at each sampling time point by means of pHmeter Beckman Instrument mod Φ 350 (Furlenton, CA, USA) and Hamilton glass electrode (Bonaduz, Switzerland).

The procedure for isolation and purification of free EPS previously reported by Mende (Mende et al., 2012) and Rimada and Abraham, 2003, was used with slight modifications. After adding 0.7 mL 80% (w/v) trichloroacetic acid (TCA) and heating to 90°C for 15 min, samples were cooled in ice water and centrifuged (2000 rpm, 20 min, 4°C) to remove cells and protein. After neutralization of the supernatant with NaOH, the EPS were purified following the procedure reported by Rimada and Abraham, 2003, and the quantification of the polymers was determined as polymer dry mass (PDM) (Van Geel-Schutten et al., 1999). Samples were centrifuged (20 min at 2600 rpm), and the pellets were dried at 55°C. EPS amount was determined by measuring final dry weight using precision analytical balances (Acculab, Bradford, MA, USA).

4.2.4 Study of the expression of the genes involved in the *Lactobacillus delbrueckii* subsp. *bulgaricus* 2214 EPS production in SSM

At each sampling time point (Fig.3), total genomic DNA was extracted using the DNeasy Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The DNA concentration was measured using an Eppendorf BioPhotometer Plus instrument and checked by agarose gel electrophoresis. The genomic DNA was diluted to 20ng/μL for PCR.

Prior to RNA was extraction cells pellets obtained after centrifugation (10min at 10,000 rpm) were grinded with liquid nitrogen using mortar and pestle. The RNA was extracted using the RNeasy Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The RNA, quantified using a spectrophotometer (Jasco, Japan) and checked by agarose gel electrophoresis, was reverse transcribed into cDNA with Quantiscript Reverse Transcriptase (QuantiTect Reverse Transcription Kit, Qiagen, Milan, Italy) using random hexamer primers according to the manufacturer's instructions.

The *epsE* gene, a phospho-glucosyltransferase from the *gtf* region of *Lactobacillus 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 found an high genetic similarity of 98% with four *Lactobacillus 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. The forward primer Ld *epsE* for CTGAGAAGCTGAAGAAGGATCTG and the reverse primer Ld *epsE* rev AGTGACATATTCCCAATCAGCAC was identified in the coding region, which yielded an amplification product of 140bp.

The partial fragments of the *epsE* gene of *L. bulgaricus* 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), 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 (vers. 4.15, Lynnon Biosoft Company).

Relative quantification was performed using the QuantStudio 3 (Applied Biosystems, Carlsbad, California, USA) with the Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, California, USA). The 20 µl PCR reaction included 1 µl of cDNA, 0.5 µl of reverse primer, 0.5 µl of sense 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 16 S rRNA as a reference genes (Table 1). 16S rRNA primers were designed for the amplification of the *Lactobacillus delbrueckii* subsp. *bulgaricus* species. The fold change at T1, T2 and T3, were obtained with respect to the T0 as a sample calibrator using the DDCT method (Livak and Schmittgen , 2001).

Table 1. Primers and PCR conditions used to detect genes related to EPS synthesis in *Lb. delbrueckii* subsp. *bulgaricus* 2214 strain.

Gene	Primer sequence	Annealing Temp.	Amp. size (bp)	References
Ld epsE for	CTGAGAAGCTGAAGAAGGATCTG			
Ld epsE rev	AGTGACATATTCCCAATCAGCAC	58	140	This study
TBA- FW	CGG CAA CGA GCG CAA CCC			Denman, S. E., and
TBA-RV	CCA TTG TAG CAC GTG TGT AGC C	63	130	C. S. McSweeney. 2006.

The real-time PCR amplification efficiencies (E) in the exponential phase were calculated according to the equation: $E=10(-1/\text{slope})$. The results showed that the amplification efficiencies were 91% for *epsE* and 92% for 16S rRNA, with a difference of less than 5% between these genes.

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

Exopolysaccharides (EPS) produced at the sampling time T0, T1, T2 or T3 by *L. bulgaricus* 2214 were inspected using both confocal laser scanning microscopy (CLSM) and 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) to observe mannopyranosyl and α -glucopyranosyl of EPS, or 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (Sigma-Aldrich) to observe bacterial cells. The staining was performed as follows: stocks solution of ConA (5 mg ml⁻¹) and DAPI (1 mg ml⁻¹) were prepared in 0.1 M sodium bicarbonate (VWR, Milan, Italy) and milliQ water, respectively. The samples (250 μ l) were added with 50 μ l of ConA and 15 μ l of DAPI in an eppendorf tube, mixed and incubated for 30 minutes at room temperature prior to CLSM observation. The EPS microstructure was analysed using an inverted CLSM from Leica

Microsystem (Heidelberg, Germany). ConA was excited at a wavelength of 488 nm using an argon laser and the emission filter was set at 515-545 nm. DAPI was excited at a 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. Numbers of counted bacteria are the mean of three different measurements.

Specimens for TEM were prepared using the conventional negative stain procedure. 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.

Two samples from each sampling time were examined by both CLSM and TEM. Several microscopic fields were observed and representative images have been selected.

4.3 Results

4.3.1 Study of the impedometric curves

The EPS+ strains impedance curves, showed a different shape as compared with the curves described in the previous work (Bancalari et al., 2016) where the original impedometric curves and fitted ones overlapped (Figure 1A). In contrast to what had been previously observed, after some hours of incubation, the E% value decreased (Figure 1B) and the two curves did not overlap anymore.

To quantify the decreasing of E % after the maximum value, the drop entity of the impedometric curves was arbitrarily expressed by the parameter $\Delta E\%$, given by the difference between the maximum value of E% and E% value after 55h of incubation (Figure 1B). The $\Delta E\%$ values

obtained for the 24 strains of this study, tested in both MRSS and SSM are reported in Table 2. The greater the $\Delta E\%$ value, the higher the slope of the curve. In MRSS, a positive increment of the $\Delta E\%$ value was observed for all the strains but to a different extent. In this case, in the measurement cells, a gelatinous biofilm around the electrodes was observed (Figure 2).

Figure 1 (A) Example of original and fitted impedometric curves. Bancalari et al., 2016 reported that if the two curves overlapped, the curves can be translated into three objective parameters using the Gompertz equation **(B)** Example of EPS+ strains impedance curves. After some hours of incubation, the E% value decreased (Figure 1B) and it was not possible to translate the curves. The drop entity of all EPS+ strains impedometric curves was arbitrarily calculated by the parameter $\Delta E\%$, which is the difference between the maximum value of E% recorded and E% value at 55h of incubation.

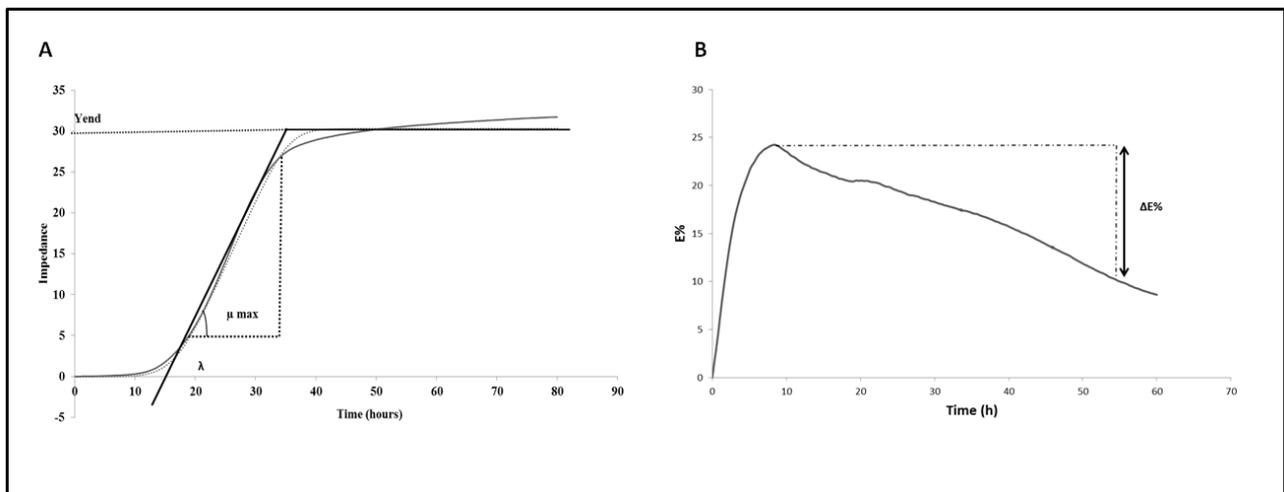
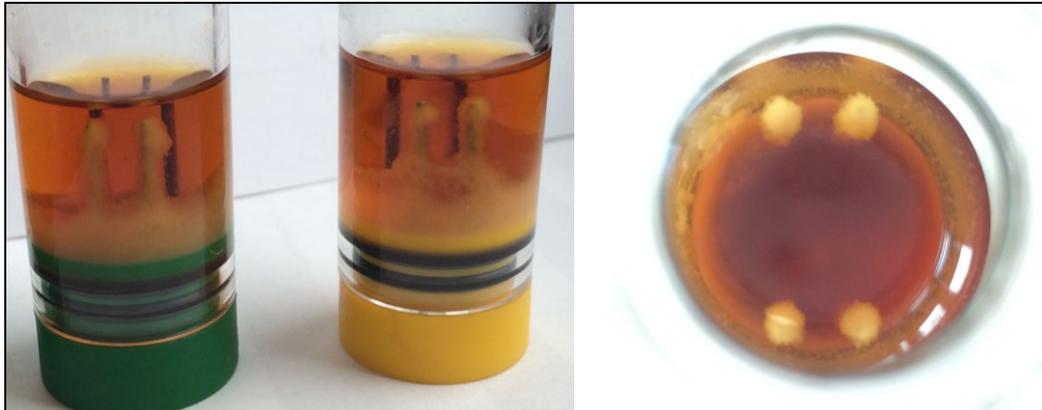


Figure 2. Macroscopic appearance of mucoïd EPS formed by *Lb. bulgaricus* 2214 in MRSS, and deposited on the surface of the electrodes in the Bac Trac measurement cells.



As expected, a negative $\Delta E\%$ value in MRSS was observed only for the EPS- *Streptococcus thermophilus* 530 that was chosen as a negative control (Table 2).

The positive $\Delta E\%$ value was variable among the strains and in particular the *Lb. bulgaricus* 2214 together with *Streptococcus thermophilus* 111 showed the greatest value. On the other hand, *Leuconostoc citreum* 4454 and 4461 together with *Lactobacillus casei* 334 showed the lowest positive value of $\Delta E\%$ (Table 2).

When the same strains were analyzed in SSM, the $\Delta E\%$ values was positive only for the 43% of the strains. This probably happened because the strains with the negative $\Delta E\%$ were not able to produce EPS in SSM, in accordance to as observed by Bauer et al., 2009, who has observed as only a limited number of microorganisms produced EPS using lactose. Following this hypothesis, only the strains with positive $\Delta E\%$, were able to utilize lactose and produce EPS in SSM. Among them, *Lb. delbrueckii* subsp. *bulgaricus* strains 2214, showed the highest $\Delta E\%$ value (Table 2), thus it was chosen as model strain for the following steps.

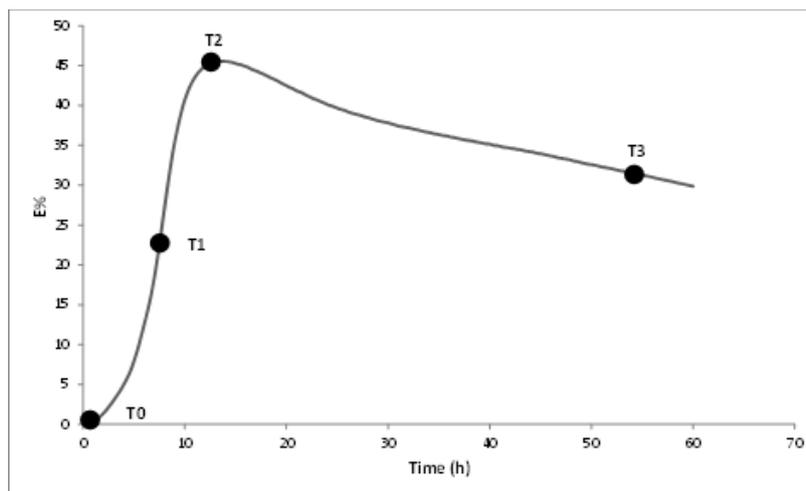
Table 2. Mean values of $\Delta E\%$ for the 24 strains tested in MRSS and SSM. All the analysis were carried out in triplicate.

	Strains	EPS	$\Delta E\%$ ^a	
			MRS+S	SSM
<i>Streptococcus thermophilus</i>	83	+	16.01±0.19	-0.07±0.02
<i>Streptococcus thermophilus</i>	111	+	23.65±0.47	-0.35±0.44
<i>Streptococcus thermophilus</i>	113	+	9.55±0.46	-0.24±0.17
<i>Streptococcus thermophilus</i>	114	+	15.47±0.49	-0.24±0.23
<i>Streptococcus thermophilus</i>	145	+	20.70±0.5	3.78±0.50
<i>Streptococcus thermophilus</i>	159	+	12.58±0.54	-0.04±0.35
<i>Streptococcus thermophilus</i>	161	+	11.84±0.46	-0.27±0.26
<i>Streptococcus thermophilus</i>	192	+	18.92±0.50	-0.37±0.12
<i>Streptococcus thermophilus</i>	530	-	-0.04±0.05	-0.04±0.05
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	1932	+	15.24±0.37	13.15±0.50
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	2214	+	24.12±0.41	18.34±0.44
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	1870	+	10.88±0.41	0.05±0.15
<i>Lactobacillus casei</i>	ATCC 334	+	4.90±0.36	-10.31±0.47
<i>Lactobacillus casei</i>	4339	+	17.42±0.14	0.83±0.31
<i>Lactobacillus paracasei</i>	4340	+	18.91±0.37	0.01±0.22
<i>Lactobacillus paracasei</i>	4341	+	19.28±0.37	-13.28±0.42
<i>Lactobacillus paracasei</i>	4366	+	22.83±0.35	-10.93±0.14
<i>Weissella</i>	4451	+	16.53±0.45	15.49±0.31
<i>Weissella</i>	4458	+	18.07±0.46	1.51±0.24
<i>Leuconostoc</i>	4454	+	7.07±0.47	-26.78±0.49
<i>Leuconostoc</i>	4461	+	8.01±0.24	-24.14±0.46
<i>Lactobacillus plantarum</i>	LMG 18399	+	22.24±0.20	-6.86±0.30
<i>Lactobacillus helveticus</i>	Lh 23	+	14.53±0.45	4.87±0.48
<i>Lactobacillus helveticus</i>	Lh 28	+	14.25±0.49	4.74±0.27

4.3.2 Impedometric curve and EPS production of *Lactobacillus delbrueckii subsp. bulgaricus* 2214

At each sampling time (Figure 3), the following analyses have been carried out: i) pH measurement, ii) expression of the genes involved in the EPS production, iii) amount of EPS (PDM) and iv) EPS characterization by confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM).

Figure 3. Impedometric curve of *Lb. delbrueckii subs bulgaricus* 2214, with the four sampling time point chosen for the analysis curves, T0 (0 hours), T1 (8 hours), T2 (13 hours) and T3 (55 hours).



The pH value was measured as an indicator of the metabolic activity, homolactic fermentation, of *L. bulgaricus* 2214. pH dropped from 6.3 to 4.5 in the first 8 hours of incubation, 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). Thus the impedometric curve reflects the microorganism growth curve, T1 correspond to the exponential growth phase (Bancalari et al., 2016). In the following time points,

the *epsE* gene expression gradually decreased. The total EPS amount, 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 55h (Table 3).

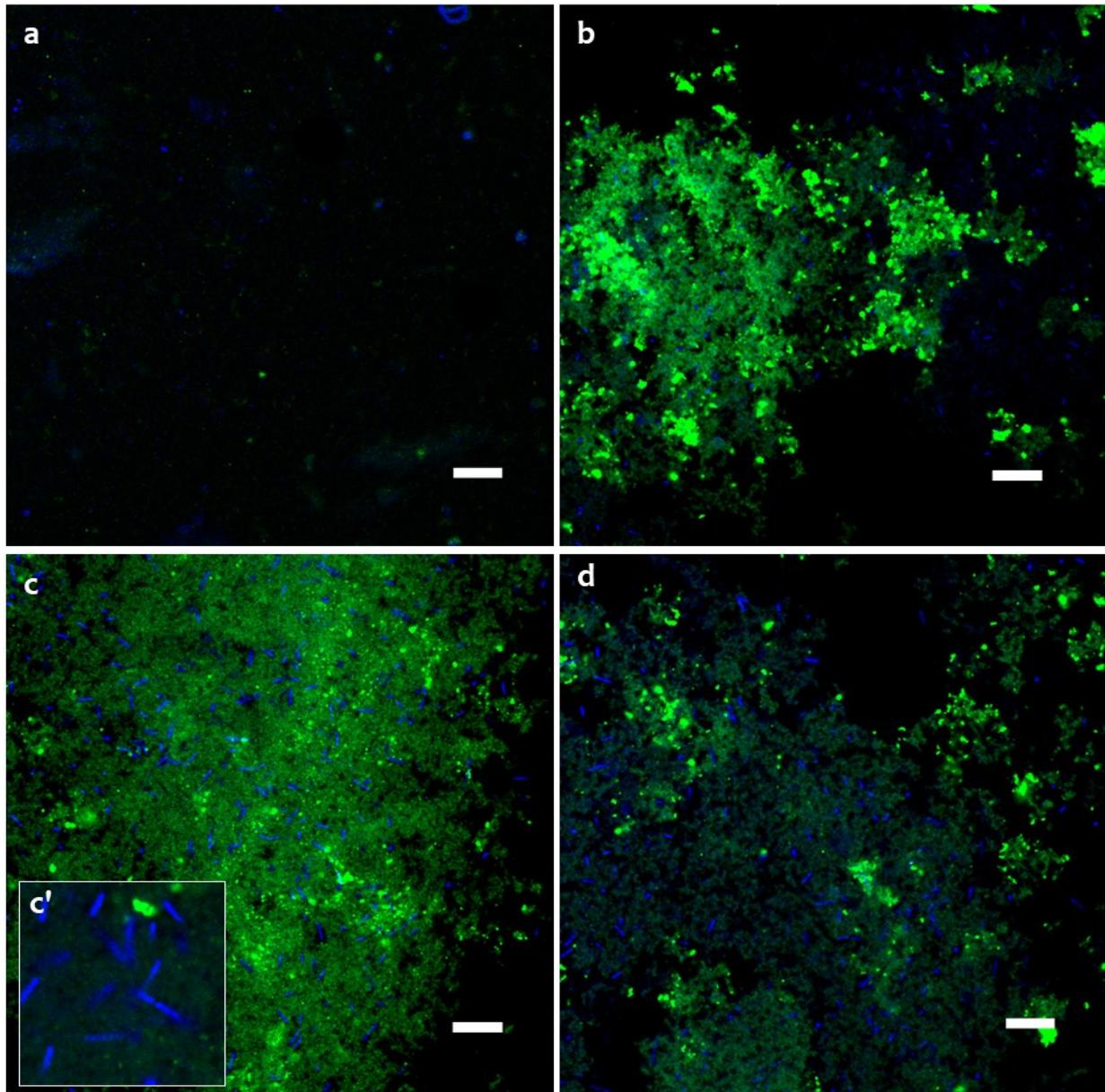
Table 3. Mean value of quantified EPS as polymer dry mass (PDM), pH and Rq, referred to each sampling time-point of *Lactobacillus delbrueckii* subsp. *bulgaricus* 2214, EPS+ strain.

Time	PDM (g/L) ^a	pH ^a	Rq ^a
T0 (0h)	0.88 ± 0.01	6.3 ± 0.0	0.005 ± 0.002
T1 (8h)	3.60 ± 0.05	4.5 ± 0.0	1.0 ± 0.035
T2 (13h)	4.64 ± 0.06	3.9 ± 0.0	0.16 ± 0.036
T3 (55h)	2.87 ± 0.05	3.4 ± 0.0	0.005 ± 0.002

4.3.3 Confocal laser scanning microscopy and transmission electron microscopy of *Lactobacillus delbrueckii* subsp. *bulgaricus* 2214

Few isolated *Lb. bulgaricus* 2214 cells were observed at T0 whereas colonies were present at subsequent time points (Fig. 4). 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. EPS emitting bright green fluorescence was detected at all points, except for T0. Volume of EPS and its fluorescence 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).

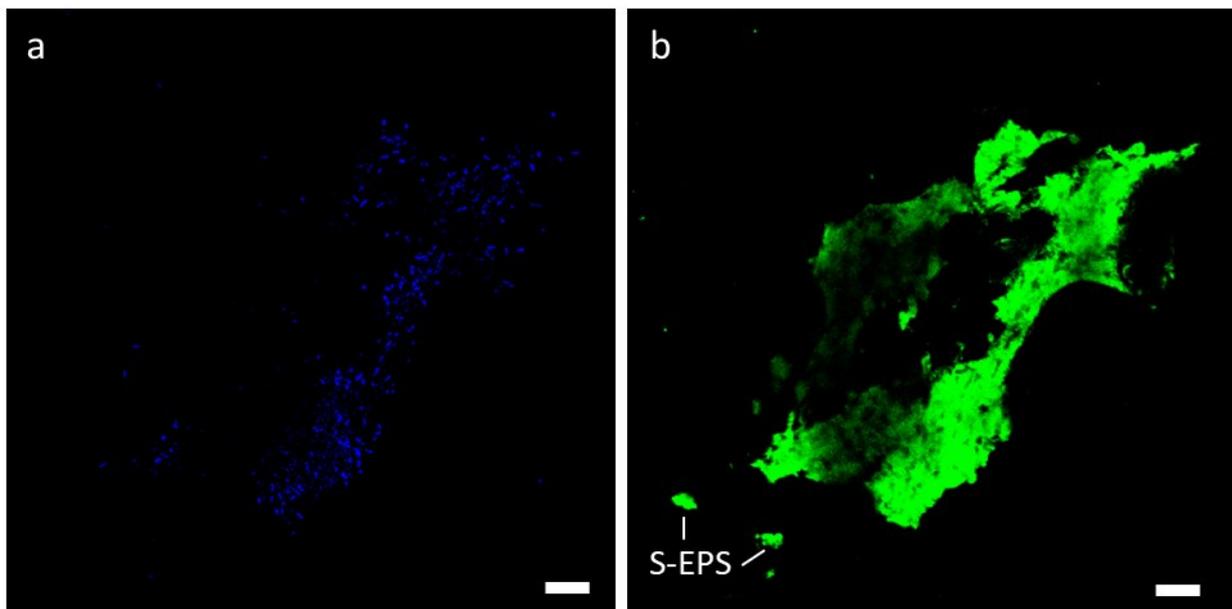
Figure 4. CLSM of *Lactobacillus delbrueckii* subsp. *bulgaricus* 2214 (a) just after inoculation in milk (T0), and after (b) 8 hours (T1), (c) 13 hours (T2), and (d) 55 hours (T3) of incubation. Bacteria labelled with DAPI appear in blue and EPS labelled with ConA appear in green. Enlarged detail of bacteria within the EPS matrix at T2 is shown in panel c'. Scale bars are 10 μm in length.



Interestingly, the combination of fluorescence probes here adopted for staining either EPS or bacteria allowed us to highlight that they exactly overlapped (Fig. 5). This indicates that cells were trapped within the EPS matrix. Small particles of EPS free of cells were also observed (Fig. 5B),

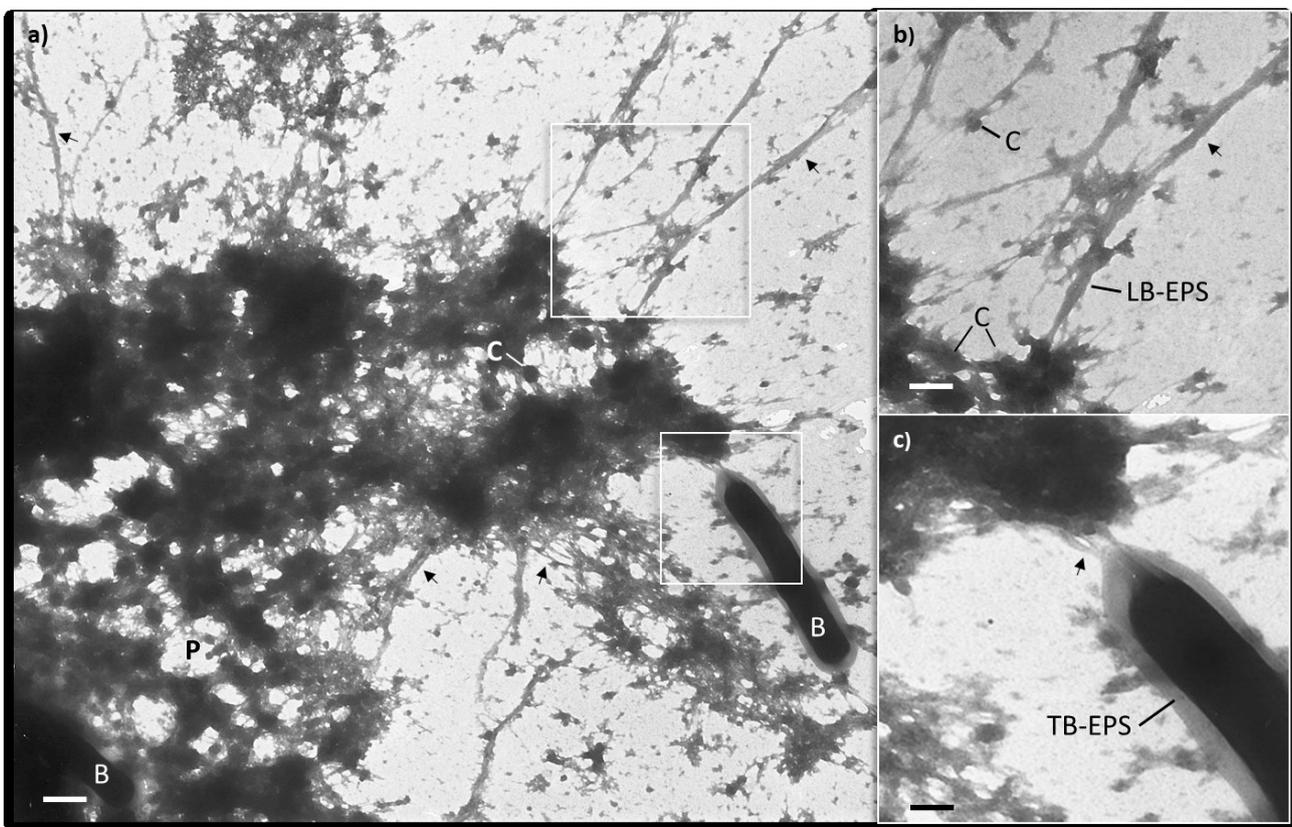
consistently with the presence of soluble EPS reported by Nielsen and Jahn,1999. Soluble EPS are poorly studied so far, the majority of the information being related to the bound EPS (Sheng et al., 2010).

Figure 5. CLSM of *Lactobacillus delbrueckii subsp. bulgaricus* 2214 and EPS in milk 13 hours after the inoculation (T2). Split channels showed (a) bacteria 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.



The milk sample at T2 was analysed also by TEM. The observation at ultrastructural level confirmed that bacterial cells are surrounded by EPS through which they bind to the protein (Fig. 6). Numerous casein micelles were trapped within the EPS network creating a highly electron-dense coarse structure. Two types of EPS were clearly distinguished in Fig. 6B, C: these could be the tightly bound and the loosely bound EPS. The former was stuck to the cell surface as a smooth layer, the latter was rather present as filaments elongating from the bacterial cell and likely used to bind to the protein matrix.

Figure 6. TEM micrographs of EPS produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* 2214 in SSM. Negative stain with uranyl acetate showed bacterial cells (B), casein micelles (C) trapped within the EPS (arrows) network and pores (P) within the structure. Panels “b” and “c” are enlarged areas of the white frames in panel “a”. Loosely bound EPS (LB-EPS) interacts with casein micelles while the tightly bound EPS (TB-EPS) forms 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”.



4.4 Discussion

Impedance microbiology is a rapid method that enables qualitative and quantitative tracing of microorganisms by measuring the change in the electrical conductivity. It is based on a principle that dates back to 1899 (Stewart, 1899) but its application in the food microbiology field is quite recent and mainly associated to the rapid detection of foodborne pathogenic bacteria (Yang and Bashir, 2008). Recently, the metabolic significance of the impedance curves of LAB in milk has

been proposed but the possibility to apply this new approach, is related to the necessity that the original and fitted curves overlap (Bancalari et al., 2016). This was not the case of the impedance curves obtained with the strains tested in the present study. When these strains were analyzed in an MRS+S broth, it was possible to observe a strain-dependent amount of EPS deposited at the surface of the measurement cells electrodes of A Bac Trac 4300[®] Microbiological Analyzer system (Figure 2). On the contrary, the electrodes were free from EPS in the case of the EPS- strain *Streptococcus thermophilus* 530, which generated a conventional impedometric curve. With this evidence, our hypothesis that the EPS production could be the cause of the descending impedometric curves was arbitrary measured by the use of $\Delta E\%$. This parameter, even if variable, was always positive when the EPS+ strains were cultivated in MRS+S. This variability could be linked to the different quantity of EPS produced or to a different chemical composition of the polymers. This aspect has not been clarified yet, but it leaves an open door to further investigate this features in a future study. When the strains were cultivated in SSM, the EPS- strain was no longer the only one able to produce a conventional impedometric curve. In fact, 10 out of 23 EPS+ strains showed a positive $\Delta E\%$ value but, differently from the MRS+S, in SSM it wasn't possible to observe the EPS deposited around the electrodes.

To investigate whether the positive value of $\Delta E\%$ in SSM was due to EPS production, three different methodological approaches were used for *L. bulgaricus* 2214 chosen as model strain. The choice to use different approaches was due to the fact that, differently from the MRS+S, the presence of EPS deposit around the electrodes is difficult to see in SSM and EPS quantification could be difficult and rather approximate (Leroy and de Vuyst, 2004).

Generally, the EPS production in milk can be evaluated by using direct analytical methods or by means of indirect measurement. In this work both indirect methods, through the study of the *epsE* gene expression, and two direct quantification methods were used.

Genes encoding EPS-biosynthesis proteins in LAB are typically organized in clusters with an operon structure, and the genes, in the *eps* operon, 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 are key enzymes for the biosynthesis of the EPS repeating units. The GTFs catalyze the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, thereby forming a glycosidic bond (Zeidan et al., 2017; Van Kranenburg et al., 1999). The GTFs in LAB are located in the central region of the eps clusters (Jolly and Stinglele, 2001).

The *epsE* gene was chosen as a 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 a lipophilic carrier (Lamothe et al., 2002). With the aim of establishing a link between gene expression and phenotype, the total amount of EPS produced was quantified as PDM. The limitation of this method is that it allows the quantification of the total polymers that are insoluble in ethanol (Goh et al., 2005). Therefore, the presence of small amounts of low molecular weight carbohydrates (e.g., lactose, glucose, etc.), could bring to EPS overestimation (Goh et al., 2005). This could be the explanation as to why in our experiments an amount (< 1 g/L) of PDM was quantified also at T0 when CLSM analysis indicate that no EPS production took place (Table 3).

The amount of EPS was 3.60 ± 0.05 g/L at T1 and increased to 4.64 ± 0.06 g/L at T2 (Table 3). This increment in quantity, despite the relative quantification of *epsE* gene expression decreased, was probably due to the fact that the EPS synthesis occurs mainly during the exponential growth, when the gene expression was greater, resulting in an accumulation of EPS at T2 (Table 3). Consistently with this observation, higher numbers of bacteria with greater production of EPS were concomitantly observed by the CSLM analysis at T1 and T2 (Fig. 4).

Interestingly, both the total EPS quantity and the relative gene expression decreased at T3. Many studies have shown decline of the EPS content during prolonged fermentation. A decrease in EPS level may be the result of : i) a physiologically changing cell environment (Gancel and Novel 1994 a, b), ii) the degradation by glycohydrolytic activity (Cerning et al. 1988, 1990, 1992; De Vuyst et

al., 1998; Pham et al., 2000), or iii) reversible DNA rearrangements resulting in different cell types with different exopolymer production capacities (Gancel and Novel 1994b). Furthermore, De Vuyst and colleagues (1998) also stated that temperature and pH could influence EPS degradation during fermentation.

The EPS produced by *L. bulgaricus* 2214 proved to have a very complex ultrastructure. We observed the presence of both EPS trapped within the protein matrix or the capsular EPS layer, tightly bound to the cell. The latter is also known as “glycocalyx”, described as polysaccharides layered on the bacteria surface together with glycoproteins (Ruas-Madiedo and Reyes-Gavilán, 2005). Hassan et al., 1995 reported the existence of *Lactobacillus delbrueckii* spp. *bulgaricus* strains able to produce both capsular and ropy EPS. About the EPS composition, we can speculate that we were dealing with hetero-EPS since, as shown by Arltoft et al., 2007, the lectin ConA has high affinity for binding to them. Our TEM observations suggested that EPS formed a network-like structure capable of entrapping protein or even small casein micelles. Also supported by SEM results of Ayala-Hernandez et al. (2008), we can state that EPS interacted with the protein matrix and were not simply located in void spaces. Such a deep interaction might explain the presence of some intact casein micelles in SSM at low pH due to the homolactic fermentation. Ayala-Hernandez et al. (2008) also demonstrated that EPS molecules may interact not only with casein but also with whey proteins, playing an active role in the formation of aggregates and thus improving the viscosity of fermented milk made by EPS-producing LAB.

In conclusion, this study has increased the understanding of the conditions determining EPS production. The impedometric approach used in this study to detect the production of EPS in milk turned out to be a useful method that allows a rapid and economic screening of a large number of samples at the same time, despite the fact that it could not give any information about the nature or the amount of the EPS produced. Since production of EPS is a very unstable feature, this method could be applicable for the study of the best *in situ* producing-conditions that are linked to different growth parameters.

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PRODUCTS

- Articles

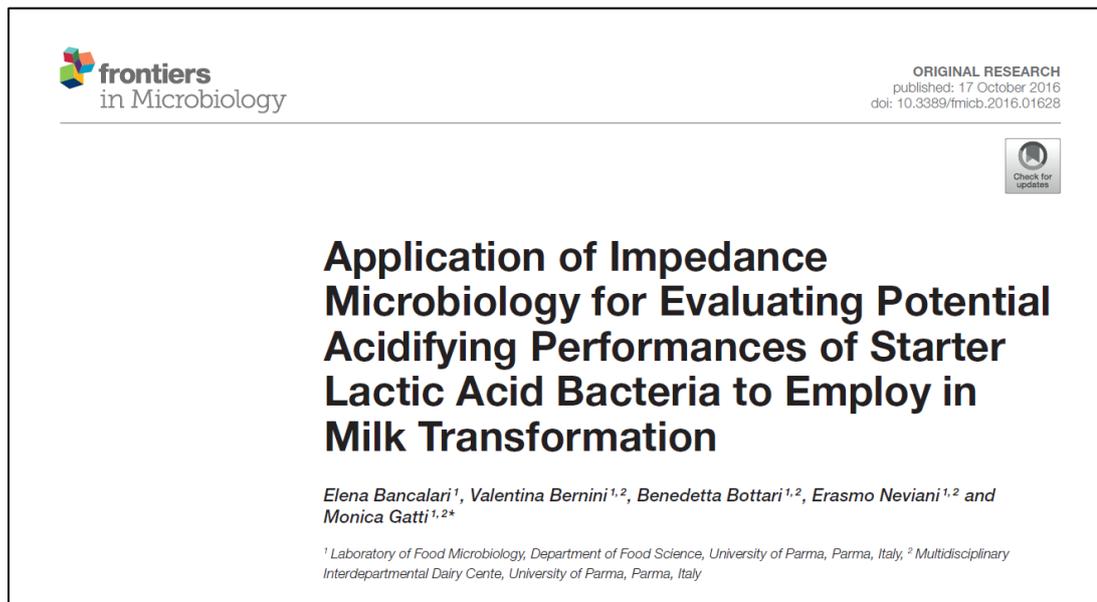
1. Ferrocino I, Di Cagno R, De Angelis M, Turrone S, Vannini L, **Bancalari E**, et al. (2015) Fecal Microbiota in Healthy Subjects Following Omnivore, Vegetarian and Vegan Diets: Culturable Populations and rRNA DGGE Profiling. *PLoS ONE* 10: e0128669. doi:10.1371/journal.pone.0128669



2. Garofalo C, **Bancalari E**, Milanović V, Cardinali F, Osimani A, Savo Sardaro ML, Bottari B, Bernini V, Aquilanti L, Clementi F, Neviani E, Gatti . Study of the bacterial diversity of foods: PCR-DGGE versus LH-PCR. (2016). *International Journal of Food Microbiology* doi: 10.1016/j.ijfoodmicro.2016.11.008



3. **Bancalari E**, Bernini V, Bottari B, Neviani E and Gatti M (2016). Application of Impedance Microbiology for Evaluating Potential Acidifying Performances of Starter Lactic Acid Bacteria to Employ in Milk Transformation. *Frontiers in Microbiology*. 7:1628. doi: 10.3389/fmicb.2016.01628



4. **Bancalari E**, Savo Sardaro M.L, Levante A, Marseglia A, Caligiani A, Lazzi C, Neviani E, Gatti M. (2017). An integrated strategy to discover *Lactobacillus casei* group strains for their potential use as aromatic starters. *Food Research International*.
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- **Oral presentation at National Congress and Workshop**

1. “Analisi impedometrica per la valutazione delle caratteristiche acidificanti di batteri lattici starter da impiegare nelle trasformazioni del latte” , **Elena Bancalari**. Universo Latte: Giovani Ricercatori e Imprese a Confronto. Piacenza, 17 – 18 marzo 2016.

Abstract

L’analisi impedometrica si basa sul principio che durante la crescita microbica avvengono processi metabolici che producono variazioni di conducibilità elettrica nel terreno di coltura, proporzionali al cambiamento del numero di microrganismi ed alla loro attività metabolica (Mucchetti et al.,1993). Tale tecnica, in passato utilizzata principalmente per il controllo qualità nell’industria alimentare, è stata utilizzata nel presente lavoro per studiare le caratteristiche acidificanti di batteri lattici (LAB) in latte. In particolare, obiettivo dello studio è stato trovare un innovativo metodo di elaborazione dei risultati, che in modo più oggettivo e standardizzabile, fosse in grado di interpretare il significato metabolico delle curve conduttimetriche.

Questo approccio consente di comparare in modo rapido e oggettivo le caratteristiche che contraddistinguono le diverse specie in esame, lavorando contemporaneamente su un grande numero di isolati e fornendo informazioni di grande interesse tecnologico. Dall’osservazione dei parametri oggettivamente descritti per tutti ceppi è possibile selezionare isolati con le caratteristiche più idonee per l’utilizzo previsto. Questo nuovo metodo di interpretazione dei risultati, potrebbe essere facilmente esteso alle diverse tipologie di strumento utilizzate per analisi impedometriche.

2. “Different methodological approaches to study lactic acid bacteria traits for their potential technological application”. **Elena Bancalari**. 22nd Workshop on the *Developments in the*

Italian PhD Research on Food Science Technology and Biotechnology, Free University of Bozen, Bozen, September 20th-22nd, 2017.

Abstract

Among the various application of lactic acid bacteria (LAB) in food processes, they are commonly used in dairy industries to acidify milk and to enhance the flavour of the end products.

In milk transformations LAB play an important role as starter (SLAB) in the acidification of milk, for fermented milk production, and in the acidification of curd, for cheese production. Furthermore, they are involved in the flavour production that is a consequence of their metabolism and is usually strain dependent. Non starter LAB (NSLAB), often used as secondary and adjunct starters for cheese production, can greatly influence flavour characteristics of the final product (Gobbetti M. et al., 2015). Then the selection criteria both for SLAB and NSLAB result very important for choosing the LAB more suitable for the intended use. Another interesting property of some strains of various genera within the LAB group is their ability to synthesize exopolysaccharides (EPS) that are extensively investigated biomolecules that exhibit heterogeneous composition, structure and broad range of physicochemical properties and application (Malang S.K. et al., 2015, Galle S. et al., 2014). EPS show a positive effect on the texture, mouthfeel, taste perception and stability of fermented foods and they could be used to replace or reduce the use of external hydrocolloids derived from plant or seaweeds that are currently in use in food industries. Recently, the increasing demand for natural polymers for industrial application, has led to a renewed interest in EPS production by LAB, especially for the *in situ* production (Leroy F., De Vuyst 2004, Kumar A.S. et al., 2007, Zannini E. et al., 2015, Salvucci E. et al., 2016). Considering all the desirable attributes that LAB can confer to food products, effort have been made to use LAB as microbial cell factories for the production of industrially interesting metabolites either to be used as purified compounds or

to be produced *in situ* in fermented foods. (Torino MI. et al., 2015, Bogouta M.A. et al., 2014, Galle S. et al., 2014). With the aim to select strains with interesting properties, in this project, different methodological approaches has been used to study LAB to be used as new, functional cultures that may lead to an improved fermentation process and an enhanced quality of the end product.

- **Posters at International Conference**

1. **Bancalari E**, Levante A, Caligiani A, Marseglia A, Neviani E, Lazzi C, Gatti M. Production in milk of volatile compounds by non starter lactic acid bacteria. *SfAM Summer Conference 2015, Fermented Foods and Beverages*. 29 June to 2 July 2015 Intercontinental Dublin, Ireland.
2. Garofalo C, **Bancalari E**, Milanović V, Cardinali F, Osimani A, Savo Sardaro ML, Bottari B, Bernini V, Aquilanti L, Clementi F, Neviani E, Gatti . Study of the bacterial diversity of foods: PCR-DGGE versus LH-PCR. *III International Conference on Microbial Diversity The Challenge of Complexity - MD 2015 – 27-29 OCTOBER 2015, Perugia*.
3. **Bancalari E**, Neviani E, Gatti M. The other side of impedance microbiology: how to study LAB technological features. *12th International Symposium on Lactic Acid Bacteria*. August 27th to August 31st, 2017. Egmond aan Zee, the Netherlands.
4. **Bancalari E**, Savo Sardaro M.L, Levante A, Marseglia A, Caligiani A, Lazzi C, Neviani E, Gatti M. (2017). An integrated strategy to discover *Lactobacillus casei* group strains for their potential use as aromatic starters. *4th International Conference on Microbial Diversity: drivers of microbial diversity, Bari 24-26 October 2017*.

- **Posters at National Workshop**

1. **Bancalari E.** How to evaluate the proper methodological approach to study lactic acid bacteria traits according to their potential technological application. XX workshop on the developments in the italian phd research on food science, technology and biotechnology. Perugia, september 23rd-25th, 2015.
2. **Bancalari E.** Acidification and aroma formation: criteria for the selection of LAB strains useful for milk transformations. 21st Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, University of Naples Federico II, Portici, September 14th-16th, 2016.

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Elena Bancalari