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Disruption treatments on two strains of *Streptococcus thermophilus*: Levels of lysis/permeabilisation of the cultures, and influence of treated cultures on the ripening profiles of Cremoso cheese

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ABSTRACT

The influence of several disruption treatments on the viability and the release of intracellular enzymes of two strains of *Streptococcus thermophilus* (St1 and St2) was studied. In addition, the impact of the incorporation of disrupted cultures of St2 in Cremoso cheese was investigated. Enzymatic (mutanolysin) and mechanical treatments (bead mill and ultrasound) were the most effective for lysis, while chemical methods (ethanol and SDS) were effective as permeabilising agents. Physical methods (freeze and heat shock) were not suitable for disruption. The application of disrupted cultures of St2 in Cremoso cheese led to slight changes on the ripening profiles: decrease in lactose level, and increase in total free amino acids and acetic acid. This work demonstrated that certain types of disrupted St2 cultures could be suitable as source of key enzymes for the production of food with improved properties, such as lactose-hydrolysed dairy products or cheeses with higher proteolysis.

1. Introduction

Lysis and permeabilisation methods are useful to prepare disrupted lactic cultures (viable or non-viable), which have been proposed for their use in foods, such as cheese (Aly, 1994; Smith, Browning, & Pawlett, 2003) and low-lactose milk products (Somkuti, Dominiecki, & Steinberg, 1998), since they are a source of intracellular enzymes and cofactors (Upadhyay & McSweeney, 2003). Both treated and autolytic lactic cultures have been used to enhance cheese flavour. These approaches release intracellular enzymes into the cheese matrix or increase membrane permeability, which enhances enzyme and substrate contact (Smith et al., 2003; Upadhyay & McSweeney, 2003).

Over the last three decades, several treatments based on different principles (physical, chemical, enzymatic, and genetic among others) have been proposed as lysis and permeabilisation promoters (Doolan & Wilkinson, 2009; Geciova, Bury, & Jelen, 2002; Middelberg, 1995; Tabanelli et al., 2015). Although they are diverse, all the methods aim to generate controlled damage in the cellular envelopes: wall or membrane.

Mechanical treatments such as ultrasonic waves and bead mills have been applied in the disruption of *Lactobacillus delbrueckii* subsp. *bulgaricus* 11,842 for lactose hydrolysis in dairy products by Bury, Jelen, and Kaláb (2001). A more recent report used sonication to prepare a freeze-dried cell-free extract from cheese-related and nonrelated microorganisms as sources of diverse enzymes to accelerate the ripening of Pecorino-type cheese (Calasso, Mancini, Di Cagno, Cardinali, & Gobbetti, 2015). Burns et al. (2015) assayed the application of sub lethal high pressure homogenisation treatment for the production of short ripened cheese with higher quality and different volatile profile. Upadhyay, Huppertz, Kelly, and McSweeney (2007) reported that the addition of high pressure-treated starter bacteria led to an acceleration of secondary proteolysis in Cheddar cheese. Somkuti et al. (1998) used ethanol and sodium dodecyl sulphate as chemical agents for the permeabilisation of *Streptococcus thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* to increase the hydrolysis of lactose in milk by increasing the β-galactosidase activity. In addition, a enzymatic treatment with mutanolysin and lysozyme was efficient for the lysis and release of proteinase of several strains of *Lactococcus lactis* subsp. *lactis* and *S.*

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thermophilus (Coolbear, Pillidge, & Crow, 1994). Although some solvents and enzymatic methods are effective for cell lysis, they are not used in food. Apart from the methods mentioned above, some other strategies have been proposed: heat-shock, freezing/thawing, and freeze or spray drying (Klein & Lortal, 1999; Madkor, Tong, & El Soda, 2000).

Most studies on lactic culture disruption and its applications address *Lactococcus lactis* (Doolan & Wilkinson, 2009; Hannon et al., 2003; Yarlagadda, Wilkinson, O'Sullivan, & Kilcawley, 2014) as it is the most widely used starter culture in the world. *S. thermophilus* is one of the most valuable starters that, for a long time, has been used for the production of fermented dairy products (Cui et al., 2016). In Argentina, it is widely used for the production of soft and semi-hard cheese (Bude-Ugarte, Guglielmotti, Giraffa, Reinheimer, & Hynes, 2006); in fact, the acidification in Cremoso cheese is led only by *S. thermophilus* (López Morales et al., 2018). The information available on disruption of *S. thermophilus* is much more limited than that for *Lc. lactis*, despite its enormous economic importance as a lactic culture: it is the second most common lactic starter worldwide. Research on *S. thermophilus* has been addressed in relation to improving the flavour in cheese (Kebary, Salem, Hamed, & El-Sisi, 1997; Smith et al., 2003) and improving health issues such as lactose intolerance (Somkuti, Dominiecki, & Steinberg, 1996; Somkuti et al., 1998) or carcinogenic risk derived from alcoholic beverage consumption (Zotta, Ricciardi, Rossano, & Parente, 2008).

The objective of this study was to assess the performance of different disruption methods, i.e., lysis and permeabilisation, in two strains of *S. thermophilus* (St1 and St2). For this purpose, we applied chemical and enzymatic agents and mechanical and physical methods. We also evaluated combined treatments. Also, the influence of the addition of disrupted cells of St2, the strain most affected by the disruption treatments, was studied in Cremoso cheese. Specifically, treated cultures of St2 by means of the treatments that showed the most effect on the indexes of lysis and permeabilisation: mechanical, chemical and enzymatic methods, were studied.

2. Materials and methods

2.1. Bacterial strains and growth conditions

S. thermophilus strains were isolated from commercial starter cultures proposed by leading companies for soft and semi-hard cheese making in Argentina. These were named *S. thermophilus* 1 (St1) and *S. thermophilus* 2 (St2). Each starter culture was propagated at 37 °C in Elliker broth (Biokar Diagnostics, Allonne, France) and plated on Elliker agar. The isolated colonies from the plates were inoculated in the same broth for culturing overnight. The new pure cultures were stored at −20 °C in Elliker broth containing 15% glycerol until ready for use.

2.2. Preparation of the cells for disruption

The cultures stored at −20 °C were grown twice (2% inoculum) in Elliker broth at 37 °C for 24 h. For each treatment, an aliquot of the overnight culture was inoculated (2%) in 50 mL of the same broth and incubated at 37 °C to a population density of approximately 5×10^8 cfu mL^{-1} (optical density at 560 nm, 1.8), which corresponded to late exponential growth phase. The cells were then harvested by centrifugation (12,000×*g* for 10 min at 4 °C) and washed twice with 50 mM potassium phosphate buffer, pH 7 (KPi buffer), and, depending on the treatment to be applied, were resuspended either in the same buffer (bead mills, ultrasound, heat shock and freeze shock) or in the appropriate solution (0.1% sodium dodecyl sulphate, 40% ethanol and 100 U or 200 U mutanolysin) as described below.

Untreated washed cells resuspended in KPi buffer were used as the controls in the chemical, mechanical and physical methods. For the enzymatic method, which required a longer period of incubation, the control was prepared with washed cells resuspended in KPi buffer and incubated under the same conditions as the treatment but without the addition of the enzyme.

2.3. Methods of disruption

The washed cells (WC) were exposed to different methods of disruption, which are described below. Immediately after each treatment, the samples were analysed for microbial counts and enzymatic activity, according to the methodologies described in sections 2.4 and 2.5, respectively. All methods of disruption were conducted in duplicate.

2.3.1. Chemical methods

WC were resuspended in 50 mL 0.1% sodium dodecyl sulphate at 37 °C for 30 min (designated C1) or 5 mL of a 40% ethanol solution for 30 min (C2). Immediately after incubation, the suspensions were centrifuged at 10,000×*g* for 4 min and the cell pellets were washed twice with KPi buffer to remove the chemical agents. Then, they were resuspended in 50 mL KPi buffer at 4 °C, and immediately analysed.

2.3.2. Enzymatic methods

Mutanolysin (Sigma, M9901) solutions (2.5 mL) of 100 (designated E1) and 200 U (E2) were applied to WC and incubated at 37 °C for 22 h. After treatment, the suspensions were adjusted at 50 mL with KPi buffer at 4 °C and immediately analysed.

2.3.3. Mechanical methods

Bead mill and ultrasound mechanical treatments (designated M1 and M2, respectively) were studied.

In M1, the cells of each strain were processed with a Minibeadbeater 8TM cell disrupter (Biospec Products Bartlesville, OK, USA). For this, WC were resuspended in 1.6 mL KPi buffer containing 1.2 g of glass beads (106 μm, Sigma) to provide a 30-fold concentration of the cells (Peralta, Bergamini, & Hynes, 2016) and disrupted in the bead mill three times for 1 min each with 1 min of cooling on ice after each disruption. After treatment, the suspensions were adjusted to 50 mL with KPi buffer at 4 °C. In M2, a suspension of WC in 50 mL KPi buffer was treated in an ultrasonic bath (Ultrasonic Cleaner 8892, Cole–Parmer, Illinois, USA) for 1 h at 5 °C at low frequency (40 kHz). The samples were immediately analysed after the treatments.

2.3.4. Physical methods

Heat shock and freeze shock physical treatments (designated P1 and P2, respectively) were studied. For P1, a suspension of WC in 50 mL KPi buffer was heated at 63 °C for 20 s in a thermal bath and rapidly cooled in an ice bath at ~4 °C. For P2, a similar suspension of WC was frozen at −80 °C for 48 h and then thawed at ~4 °C. After the treatments, the samples were immediately analysed.

2.3.5. Combination of the methods

We also tested combinations of the sodium dodecyl sulphate with heat shock (CP1) or freeze shock (CP2) and ethanol with heat shock (CP3) or freeze shock (CP4). The treatments were described above, and the order was chemical treatment first, followed by the physical treatment. After the treatments, the samples were immediately analysed.

2.4. Microbial counts

Counts of *S. thermophilus* were determined on the cell suspensions taken before and after the disruption treatments as detailed above. The plate counts were performed on Elliker (Biokar Diagnostics, Allonne, France) agar incubated at 37 °C for 48 h.

2.5. Enzyme activities

Two intracellular enzymes that are classical indexes of cell permeabilisation or lysis were selected to monitor the effect of the treatments: lactate dehydrogenase (LDH) and β-galactosidase (β-gal) (Bunthof, Van Schalkwijk, Meijer, Abee, & Hugenholtz, 2001; Bury et al., 2001; Daryaei, Coventry, Versteeg, & Sherkat, 2010; Wilkinson, Guinee, & Fox, 1994). Their total and released activities were analysed in the cell suspension or clarified supernatant, respectively, from samples after disruption treatment and control samples (Bunthof et al., 2001; Bury et al., 2001). The enzymatic activity of the cell suspension, which included cells, cell debris, and intracellular content released to the medium, was named "total activity"; this value represents the sum of the intracellular enzymatic activity of permeabilised cells and those released to the medium. The "released enzymatic activity" was measured in the same suspension that was previously centrifuged at 10,000×*g* for 4 min to remove the cells and cell debris; this clarified supernatant only contained the intracellular content released to the medium. The results were expressed as differences in the enzyme activity between the samples after the disruption treatments and their respective control.

In the particular case of the M1 treatment, the assessment of the total enzymatic activity was not recorded because the glass beads could not be separated from the cells and cellular debris.

2.5.1. Lactate dehydrogenase activity

LDH activity was assessed by measuring the decrease in absorbance at 340 nm resulting from the pyruvate-dependent oxidation of NADH (Daryaei et al., 2010). An aliquot of 400 μL cell suspension or clarified supernatant (for determination of total and released activity, respectively) was mixed with 1100 μL 100 mM sodium phosphate buffer pH 6.5, 200 μL 62.5 mM sodium pyruvate, 200 μL 12.5 mM D-fructose 1,6-bisphosphate, and 600 μL 0.625 mM NADH. The initial linear reaction rates were estimated from the change in absorbance at 340 nm measured immediately after the addition of sodium pyruvate. Using the molar extinction coefficient of 6300 M^{-1} cm^{-1} (for NADH at 340 nm), the activity was defined as nmol NADH oxidised during incubation and calculated as per min and μL extract.

2.5.2. β-galactosidase activity

The β-gal activity was measured according to Vinderola and Reinheimer (2003). An aliquot of 100 μL cell suspension or clarified supernatant (for the determination of total and released activity, respectively) was placed in a tube and 900 μL 100 mM sodium phosphate buffer pH 7.0, and 200 μL *o*-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma, N1127) (4 mg mL^{-1}) were added. The tubes were placed into a water bath at 37 °C for 15 min and then 500 μL 1 M Na_2CO_3 was added to each tube to stop the reaction; finally, the absorbance at 420 nm was measured in each tube. Using the molar extinction coefficient of 4500 M^{-1} cm^{-1} (for ONP at 420 nm), the activity was defined as nmol of ONP produced during incubation and calculated as per min and μL extract.

2.6. Scanning electron microscopy

The treated cultures of *S. thermophilus* that showed the most levels of lysis and permeabilisation, i.e., St2 treated with C2, C1, M2, E1, M1 methods, were assayed as adjunct cultures in miniature Cremoso cheeses (see section 2.7). To confirm the effect of these treatments on the cells of St2, untreated and treated cells were analysed by scanning electron microscopy (SEM). The untreated and treated cells were fixed with 3.5% formaldehyde in 50 mM potassium phosphate buffer, pH 7. Samples previously fixed in formaldehyde were placed on the agar surface for 2 h. The samples were dehydrated 10 min in a graded ethanol series (30, 50, 70, 90, 100%) and further dehydrated 10 min in acetone (100%) and finally dried to a critical point in a Denton Vacuum DCP 1. The samples were mounted on aluminium stubs and coated with gold Ion Sputter (JFC-1100, JEOL) and examined in a Zeiss SUPRA 55VP scanning electron microscope.

2.7. Cheesemaking

The influence of the incorporation of disrupted cultures of St2 as adjunct cultures was assayed in miniature Cremoso cheeses, manufactured at laboratory scale (2 L), according to Milesi, McSweeney, and Hynes (2008); this strain was selected because it was most affected by the treatments studied. In these cheesemaking experiments, the treated cultures of St2 used were those given by the methods that showed the most effect on the indexes of lysis and permeabilisation. For this, two cheesemaking trials were performed; in each trial, a control cheese (C), without the addition of the treated culture, and several experimental cheeses, with the addition of a treated culture of St2, were manufactured (Fig. 1).

In trial 1, two types of experimental cheeses were made: EA and EM cheeses, in which St2 cells treated with the chemical (C2, alcohol) and enzymatic (E1, mutanolysin) methods, respectively, were incorporated.

In trial 2, three types of experimental cheeses were made: EU, ED and ES cheeses, in which St2 cells treated with the mechanical (M2, ultrasound; M1, cellular disruptor) and chemical (C1, SDS) treatments, respectively, were added.

In all cases, a lyophilised culture of St 2 was used as starter culture. The cheeses were ripened at 5 °C for 60 days. Cheeses were manufactured in duplicate.

2.8. Analysis of cheeses

2.8.1. Gross composition, pH, and microbial counts

Gross composition and pH of cheeses were assessed at 3 days of ripening by standard methods: moisture, fat, protein and pH using FIL-IDF (1997, 1993, 1982) and Bradley et al. (1993), respectively. Microbial counts were analysed in cheese samples at 3, 15, 30 and 60 days of ripening by plating on Elliker agar and incubation 48 h at 37 °C.

2.8.2. Carbohydrates and organic acids

The levels of lactose, galactose and organic acids were quantified on cheese samples at 3 and 60 days of ripening by high-performance liquid chromatography (HPLC) according to Peralta et al. (2017). The HPLC equipment consisted of a quaternary pump, an on-line degasser, UV/VIS detector all Series 200 (Perkin Elmer, Norwalk, CT, USA) and a refractive index detector (Series Flexar). The chromatographic separation was carried out at 65 °C on an Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad Laboratories, Torrence, CA, USA).

2.8.3. Amino acids

Quantification of amino acids (AA) was performed on cheese samples at 3 and 60 days of ripening by HPLC according to Milesi, Wolf, Bergamini, and Hynes (2010). The HPLC equipment was the same than that used for organic acid analysis, previously described in section 2.7.2.

2.8.4. Volatile compounds

Volatile compounds were analysed from cheese samples at 60 days of ripening by solid phase micro extraction-gas chromatography according to Peralta et al. (2017). The gas chromatography system (Perkin Elmer model 9000, USA) with a HP INNOWax column (60 m × 0.25 mm × 0.25 μm) (Agilent J & W, Agilent Technologies, USA) and a FID detector set at 290 °C was used.

3. Results

3.1. Lysis and permeabilisation

3.1.1. Microbiological counts

The influence on the viability of twelve disruption treatments applied on the two *S. thermophilus* strains is shown in Table 1. The most important decrease in the cell population in individual treatments was caused by C1 and C2, which showed reductions superior to 8 log cfu mL^{-1} for both strains. The rest of the individual treatments showed a decrease ≤2 log cfu mL^{-1}, except for M1 and P2, which had different effect on St1 and St2. M1 produced a reduction of 1.9 and 2.7 log cfu mL^{-1} for St1 and St2, respectively, whereas P2 decreased the viable population of St1 and St2 by 1.2 and 4.6 log cfu mL^{-1}, respectively. The increase in the concentration of mutanolysin from 100 to 200 U did not change considerably the viability: the tested strains showed reductions of approximately 1 log cfu mL^{-1} with both concentrations of the enzyme. The combined treatments (C + P) showed the predominance of the most aggressive treatment, and the final counts after mixed treatments were similar to those obtained by treatments with C1 and C2 alone (<1 log cfu mL^{-1}).

3.1.2. LDH and β-gal

The differences in the activities of LDH and β-gal enzymes between treated samples and their respective controls are shown in Figs. 1 and 2. The C1 treatment increased the total activity of LDH and β-gal in both strains, whereas for C2, only the total β-gal increased. The enzymatic treatments with mutanolysin increased the level of both intracellular enzymes with similar amounts for total and released activity. The increase in the mutanolysin concentration from 100 to 200 U did not show a clear trend in the release of LDH and β-gal. Regarding M1, it showed the highest release of β-gal among all treatments tested, whereas it caused only a slight increase in LDH. The M2 treatment increased the level of both enzymes with similar amounts for total and released

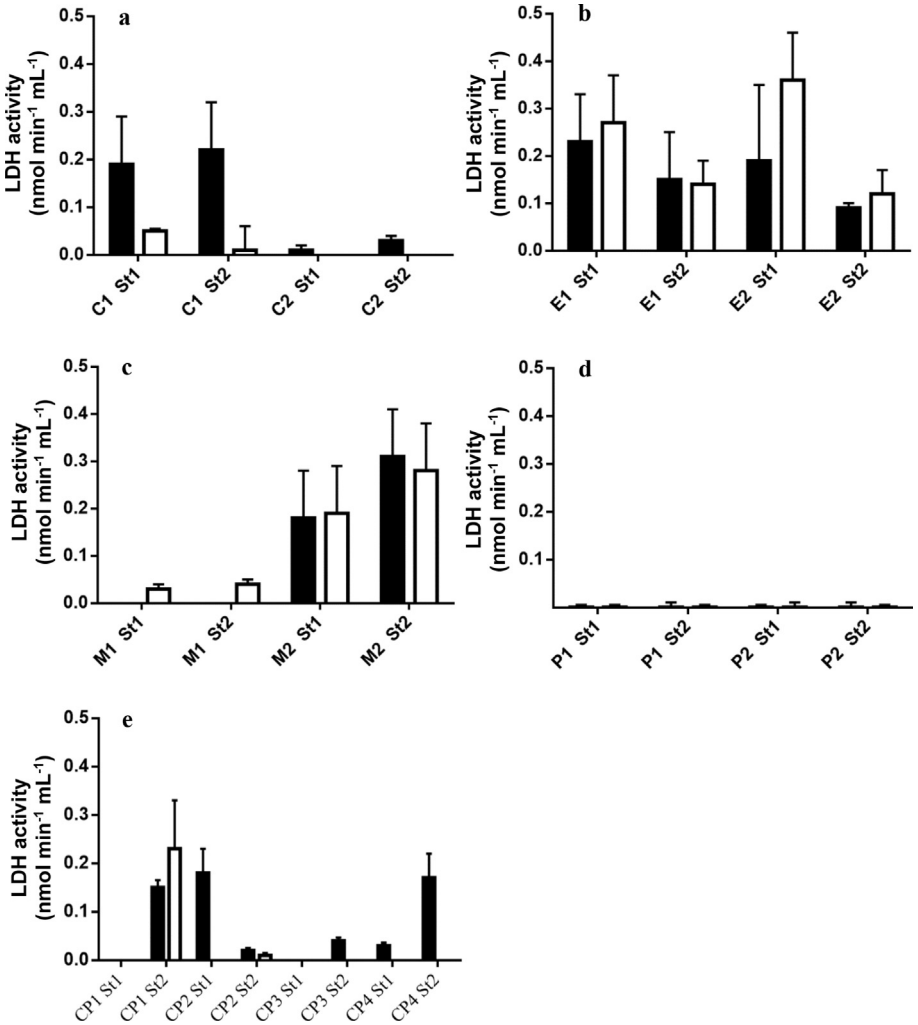

Fig. 1. Lactate dehydrogenase activity (black bar: total activity; white bar: released activity): differences in the enzyme activity between treated suspensions and their respective control after the disruption treatments (total enzymatic activities in M1 were not performed). a) Chemical treatments (C1: SDS, C2: ethanol); b) Enzymatic treatments (E1 and E2: 100 and 200 U mutanolysin); c) Mechanical treatments (M1: bead mills, M2: ultrasonic bath); d) Physical treatments (P1: heat shock, P2: freeze shock); and e) Chemical-physical (CP) treatments (CP1: C1 + P1; CP2: C1 + P2; CP3: C2 + P1; CP4: C2 + P2). Values are means ± standard deviation of the results of two independent experiments.

Table 1
Cell disruption treatments and their effects on the plate counts of two strains of *S. thermophilus*, St1 and St2.[a]

Treatment	Description	St1 cell counts (log cfu mL^{-1})		St2 cell counts (log cfu mL^{-1})	
		Before	After	Before	After
Chemical					
C1	0.1% SDS, 37 °C, 30 min	8.76 ± 0.02	<1	8.38 ± 0.01	<1
C2	40% ethanol, 37 °C, 30 min	8.79 ± 0.01	<1	8.38 ± 0.01	<1
Enzymatic					
E1	100 U mutanolysin, 37 °C, 22 h	8.82 ± 0.01	7.04 ± 0.01	8.43 ± 0.02	7.57 ± 0.01
E2	200 U mutanolysin, 37 °C, 22 h	8.82 ± 0.02	7.93 ± 0.02	8.43 ± 0.02	7.70 ± 0.02
Mechanical					
M1	Bead mills, 5 °C, 3 min	8.78 ± 0.02	6.89 ± 0.01	8.38 ± 0.02	5.70 ± 0.01
M2	Ultrasonic bath 40 kHz, 5 °C, 1 h	8.78 ± 0.02	8.00 ± 0.02	8.38 ± 0.02	8.19 ± 0.02
Physical					
P1	Heat shock 63 °C, 20 s	8.66 ± 0.01	7.63 ± 0.01	8.66 ± 0.01	7.56 ± 0.01
P2	Freeze shock −80 °C, 48 h	8.75 ± 0.02	7.60 ± 0.02	8.62 ± 0.02	4.00 ± 0.02
Chemical-Physical					
CP1	C1 + P1	8.76 ± 0.02	<1	8.38 ± 0.01	<1
CP2	C1 + P2	8.76 ± 0.02	<1	8.38 ± 0.01	<1
CP3	C2 + P1	8.79 ± 0.01	<1	8.38 ± 0.01	<1
CP4	C2 + P2	8.79 ± 0.01	<1	8.38 ± 0.01	<1

[a] Cell counts are given before and after treatments; values are means ± standard deviation of the results of two independent experiments. The combined treatments were performed under the same conditions as the individual treatments.

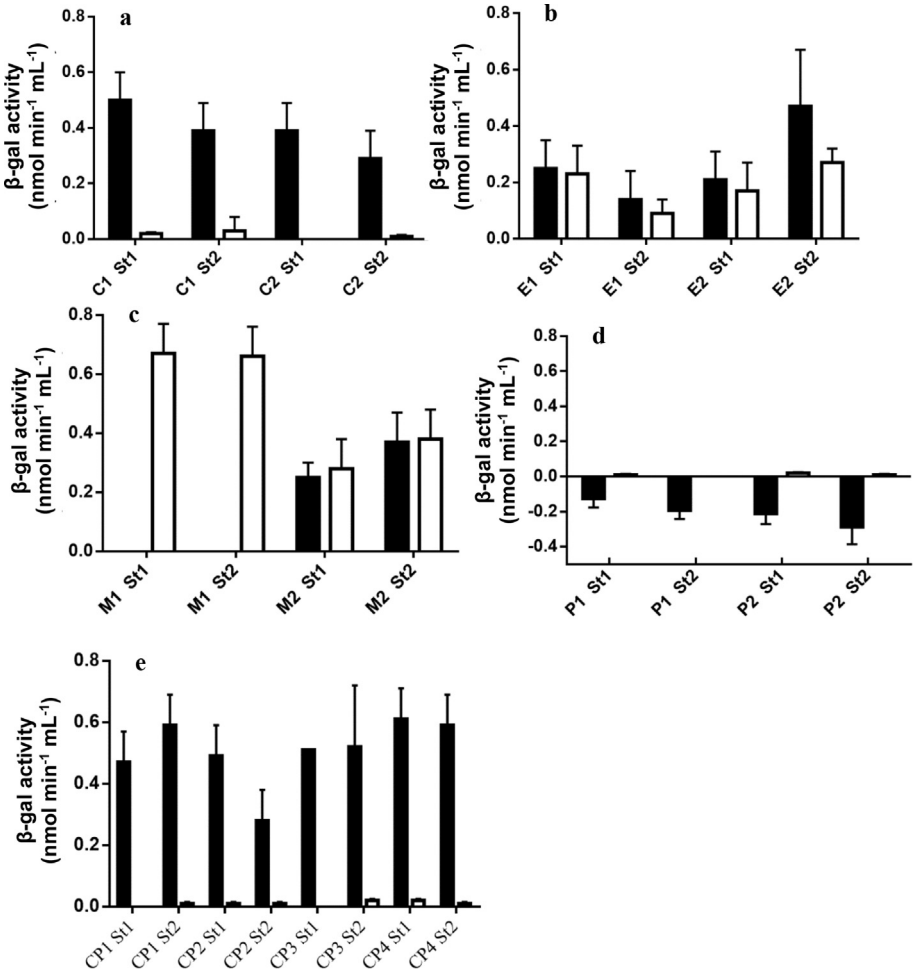

Fig. 2. β-galactosidase activity (black bar: total activity; white bar: released activity): differences in the enzyme activity between treated suspensions and their respective control after the disruption treatments (total enzymatic activities in M1 were not performed). a) Chemical treatments (C1: SDS, C2: ethanol); b) Enzymatic treatments (E1 and E2: 100 and 200 U mutanolysin); c) Mechanical treatments (M1: bead mills, M2: ultrasonic bath); d) Physical treatments (P1: heat shock, P2: freeze shock); and e) Chemical-physical (CP) treatments (CP1: C1 + P1; CP2: C1 + P2; CP3: C2 + P1; CP4: C2 + P2). Values are means ± standard deviation of the results of two independent experiments.

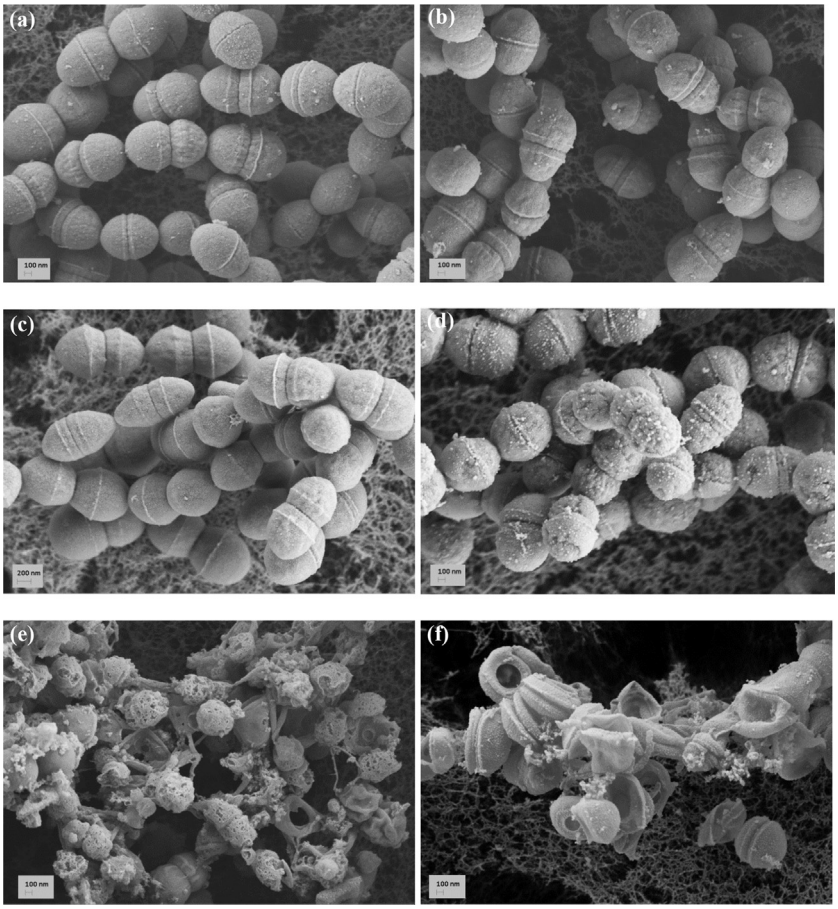

Fig. 3. Scanning electron micrographs of St2: untreated cell (a) and cells treated with C2: ethanol (b), C1: SDS (c), M2: ultrasonic bath (d), E1: 100 U mutanolysin (e), and M1: bead mill (f).

activity. P1 and P2 did not increase the activities of the enzymes tested.

Finally, the total β-gal increased in all of the mixed treatments for both strains, whereas the behaviour of LDH was variable. In effect, notable increases of total LDH were observed only in three cases (Fig. 1e), whereas an increase of the released LDH was observed only in CP1 for St2.

3.2. Scanning electron microscopy

Fig. 3 shows the micrographs obtained by SEM on St2 cells. The untreated cells are intact (Fig. 3a), while slight changes on the cell walls could be seen in the cells treated with chemical methods: alcohol and SDS (Fig. 3b,c). The ultrasonic bath greatly affected the cell walls of St2 (Fig. 3d); some deep craters and many holes in the cells were observed in these images. Finally, a wide cellular destruction was observed in the images obtained from cells after treatments with mutanolysin and bead mill (Fig. 3e,f); many completely lysed cells were found in these micrographs.

3.3. Cheeses

3.3.1. Gross composition, pH and microbiological counts

Gross composition and pH of control and experimental cheeses were similar, and the values were consistent with Cremoso Cheese (Milesi et al., 2010). The mean values were the following: pH 5.33 ± 0.10, moisture 50.79 ± 0.93% (w/w), fat 26.03 ± 0.72 (w/w), and protein 18.36 ± 0.49 (w/w). The counts of starter were about 9

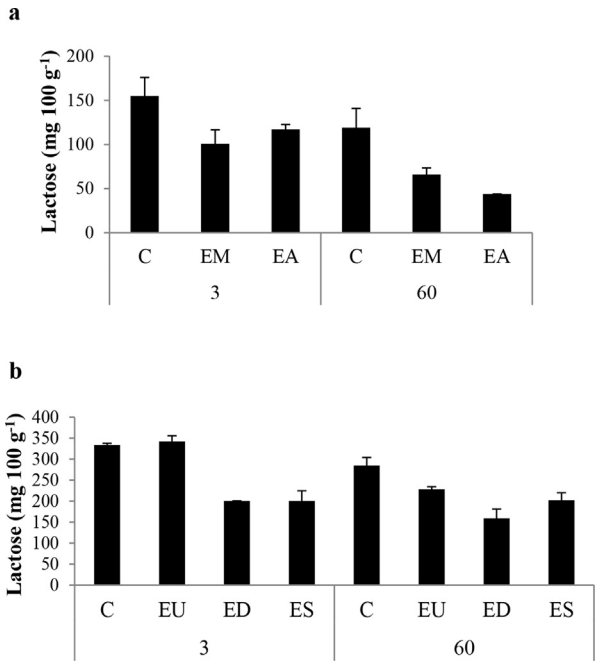

Fig. 4. Levels of lactose in the miniature cheeses from trial 1 (a) and trial 2 (b) at 3 and 60 days of ripening. Control cheese without disrupted culture (C) and experimental cheeses with addition of treated St2 cultures with alcohol (EA), mutanolysin (EM), ultrasound (EU), cellular disruptor (ED) and SDS (ES). Values (mg 100 g^{-1}) are means ± standard deviation of the results of two independent experiments.

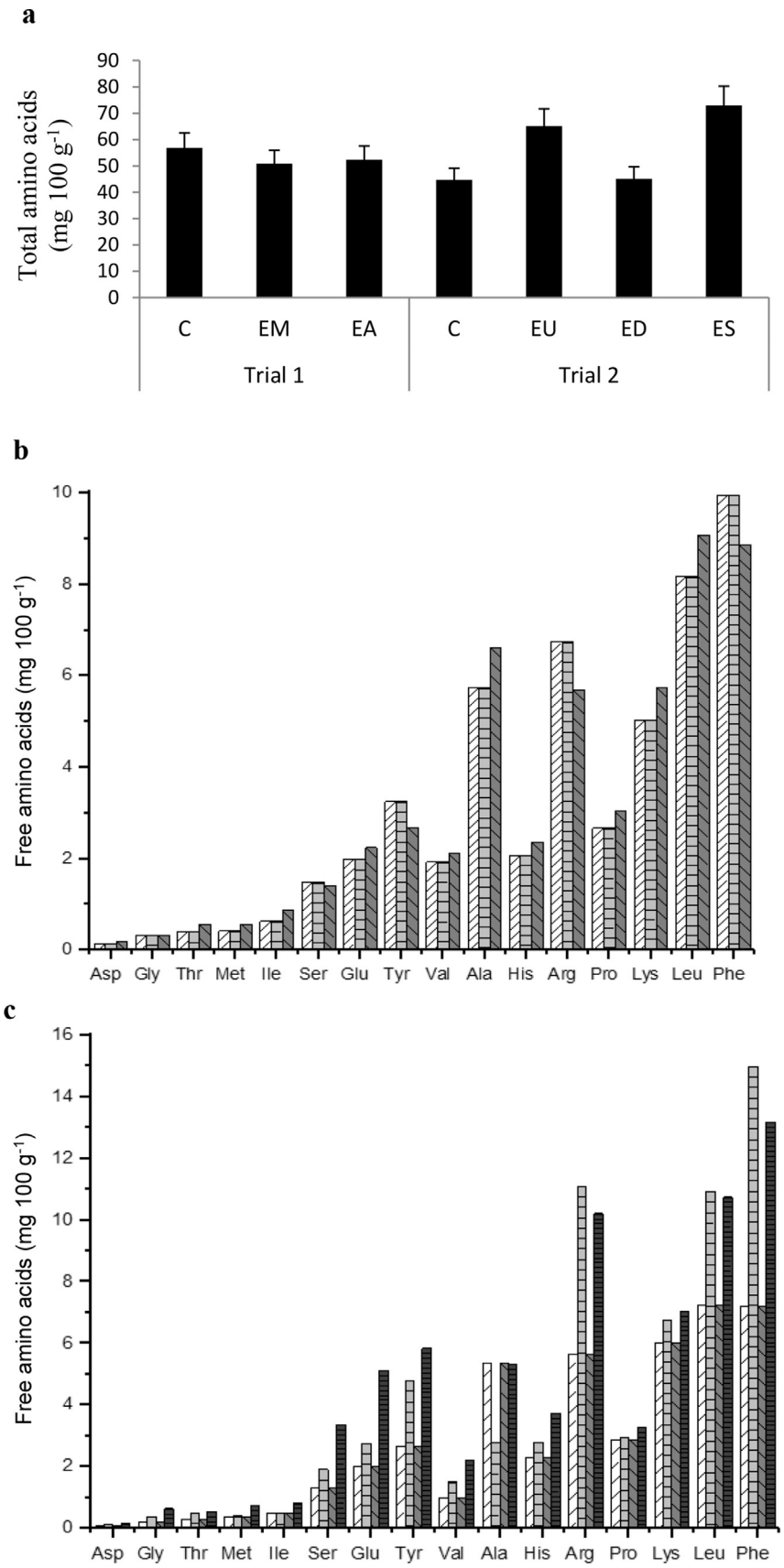

Fig. 5. Levels of total (a) and free amino acids (b, trial 1; c, trial 2) in miniature cheeses at 60 days of ripening: control cheese without disrupted culture (C, ▨) and experimental cheeses with addition of treated St2 cultures with, panel a, mutanolysin (EM, ▤) and alcohol (EA, ▩) and, panel b, ultrasound (EU, ▤), cellular disruptor (ED, ▩) and SDS (ES, ▩). Values (mg 100 g^{-1}) are means ± standard deviation of the results of two independent experiments.

log cfu g^{-1} in all cheeses; no difference was observed due to the addition of the treated cultures.

3.3.2. Carbohydrates and organic acids

The levels of lactose at 3 and 60 days of ripening were lower in all experimental cheeses in comparison with those in the control cheese, indicating an influence of the incorporation of the treated cultures (Fig. 4). Meanwhile, the levels of galactose and organic acids: lactic, citric, acetic, uric, orotic, hippuric, pyruvic and α–ketoglutaric, were similar between control and experimental cheeses (data not shown). Lactic and citric acids were found at the highest levels among the organic acids in all cheeses.

3.3.3. Amino acids

Fig. 5a shows the total AA levels in the miniature cheeses at 60 days of ripening for both trials. Noticeable differences between control and experimental cheeses were only detected in the trial 2: the addition of the treated culture by ultrasound and SDS led to an increase of the levels of the total AA in EU and ES cheeses, respectively.

The most AA (Thr, Ser, Glu, Met, Tyr, Val, Phe, Ile, Lys and Leu) increased during ripening, unlike a few ones (Ala, Asp, His, Pro and Arg) that remained almost constant (data not shown). Higher levels of some free AA, mainly Ser, Glu, Tyr, Val, Arg, Leu and Phe, were only found in experimental cheeses EU and ES in comparison with control ones at 60 days of ripening (Fig. 5b,c).

3.3.4. Volatile compounds

Twenty-two compounds were detected in the headspace of the cheeses consisting of seven alcohols (ethanol, 2-butanol, 1-propanol, 3-methyl-1-butanol, 1-pentanol, 1-hexanol, 1-heptanol), six ketones (2-propanone, 2-butanone, 2-hexanone, 2-heptanone, diacetyl, acetoin), five acids (acetic acid, butyric acid, hexanoic acid, octanoic acid, decanoic acid), three esters (ethyl acetate, methyl butanoate, ethyl butanoate) and one aldehyde (benzaldehyde) (data not shown). Marked differences between control and experimental cheeses were only found for acetic acid. The peak area of this compound was higher in the experimental cheeses than in control ones only in the trial 1.

4. Discussion

In this study, we assessed the effect of chemical, enzymatic, mechanical and physical treatments on the viability and the release of intracellular enzymes of two strains of *S. thermophilus*. The treated cells that showed higher levels of lysis/permeabilisation were used as adjunct cultures in cheese.

The chemical methods applied (C1 and C2) showed a major impact on the viability for both strains of *S. thermophilus*, which was well correlated with an increase of the total β-gal. A similar effect has been reported for *Lb. delbrueckii* subsp. *bulgaricus* and other strains of *S. thermophilus* treated with the same chemical agents (Somkuti et al., 1998; Somkuti & Steinberg, 1994). In our work, the low LDH and β-gal activities detected in the supernatant (released activity) were expected as the cells were washed to remove the chemical agent, whereas the low level of total LDH after C2 probably revealed the inactivation of the enzyme due to this treatment. Exterkate (2006) and Somkuti et al. (1998) observed that the activities of intracellular enzymes quantified as indexes of cell permeabilisation increased with the concentration of the permeabilising agent up to a limit and after which they decrease, which is probably due to chemical denaturation.

The enzymatic methods (E1 and E2) had an intermediate effect on St1 and St2; they produced a relatively low decrease in the cell viability but caused a considerable increase in the total and released activities of the assessed intracellular enzymes. Our findings for St1 and St2 are similar to those of Bunthof et al. (2001) on *Lc. lactis* subsp. *lactis* MG1363 after a treatment with 100 U of mutanolysin. Coolbear et al. (1994) obtained degradation of the cell wall of five *S. thermophilus* strains by a combination of lysozyme and mutanolysin. Enzymatic treatments have also been applied to release intracellular materials of *S. thermophilus* cells such as DNA (Chapot-Chartier, Rul, Nardi, & Gripon, 1994) or phage particles (Husson-Kao et al., 2000); however, there are no previous reports regarding the release of LDH and β-gal from *S. thermophilus* by mutanolysin treatments.

As for the mechanical methods (M1 and M2), they were effective in increasing enzymatic activities; however, they had a minimal impact on the viability compared with chemical methods and were similar or lower than those achieved with the enzymatic treatments. It is important to remark that the thermal effects of the mechanical methods were minimised; the treatment M1 was carried out at 5 °C, while during M2 treatment, cooling on the ice were applied between each cycle of disruption. The variation in enzyme activities may result from the milling cycles and the enzyme studied. Bury et al. (2001) showed that the maximum β-gal activity was released when milling between 2 and 3 min and after which the activity decreased. Hummel and Kula (1989) successfully measured LDH after cell milling in several species of lactobacilli (*Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus plantarum*) and yeast (*Saccharomyces cerevisiae*), whereas in our work, LDH recorded after milling was very low and suggested the negative impact of M1.

M2 showed a comparatively minimal effect on the viability of St1 and St2 (the diminution of cell counts was below 1 log cfu mL^{-1}), which was in agreement with studies on other species of LAB including *Lc. lactis* (Doolan & Wilkinson, 2009), *Lb. delbrueckii*, *Lactobacillus helveticus* and *Lactobacillus acidophilus* (Bury et al., 2001; Sakakibara, Wang, Ikeda, & Suzuki, 1994; Wang & Sakakibara, 1997). On the other hand, several studies have focused on increasing the β-gal activity of LAB cultures by sonication as a strategy to obtain low-lactose food products (Bury et al., 2001; Kreft & Jelen, 2000; Kreft, Roth, & Jelen, 2001). Among them, Bury et al. (2001) and Kreft and Jelen (2000) obtained positive results when using a probe sonicator, whereas our results were comparable using an ultrasonic bath. Both methods of sonication have effects on the bacteria inactivation and declumping; however, the scale of these effects depends on the intensity and frequency of the ultrasonic waves (Joyce, Al-Hashimi, & Mason, 2011; Joyce, Phull, Lorimer, & Mason, 2003). In addition to the type of sonicator, the type of bacteria is important as large bacteria (bacilli/rod) are more sensitive to sonication than small bacteria (cocci/spherical) because of the large surface (Joyce et al., 2011). Although high power ultrasound are widely used to lyse cells, the low power ultrasound, such as those from ultrasonic baths, can enhance the disruption of biological cell walls to facilitate the release of their intracellular contents (O'Donnell, Tiwari, Bourke, & Cullen, 2010). To date, the information regarding the effect of ultrasonic waves using an ultrasonic bath on the viability of bacteria is limited (Joyce et al., 2011). This is the first report on its application on the release of β-GAL from *S. thermophilus*.

Heat or freeze shocked cultures of *Lb. delbrueckii* var. *helveticus* and other LAB have been proposed for accelerated flavour production and an improved body of cheese (Aly, 1994; El Soda, Madkor, & Tong, 2000) but little is known about the enzymatic activities after thermal treatment. In this work, heat shock (P1) showed a slight reduction of viability in both strains but no increase in the enzymatic activity; on the contrary, the β-GAL activity decreased after the treatment and indicated possible inactivation.

Freeze shocking (P2) produced a notable decrease in the viability of the St2 strain; however, this effect was less pronounced for the St1 strain. A variation in the resistance of LAB to freezing and frozen storage has been observed. Some treatments could maintain cell counts after freezing such as freeze shock at −196 °C for 0.5 min for *Lc. lactis* (Doolan & Wilkinson, 2009). El Soda et al. (2000) reported a decrease of 1–2 log cfu mL^{-1} for a strain of *Lb. helveticus* and for two strains of *Lb. casei* after freeze shock at −24 °C for 40 h. In our work, slow freezing affected St2 (as expected) but St1 was more resistant. The freezing did not increase the activity of LDH and β-GAL. In fact, β-GAL decreased after P2.

The scanning electron micrographs of treated St2 cells are in agreement with the results of LDH and β-gal activities. The images of St2 treated by ultrasound and bead mill revealed similar changes than that reported by Bury et al. (2001) in cells of *Lb. delbrueckii*. These authors observed an extensive cellular destruction after processing with the bead mill, while the ultrasound had less impact, observing more intact cells.

Finally, when we studied treatments with more than one factor, we generally observed that the most aggressive treatment led the changes experienced by the culture regarding viability and enzyme activities. Some exceptions were observed for the levels of total and released LDH. First, the total LDH activity increased when the C1 treatment was applied on St1 and St2 but not in the mix treatment of CP1 (C1 + P1) on St1 and CP2 (C1 + P2) on St2, which probably suggests an inactivation of the enzyme by the physical treatment. Second, there was an increase of total LDH in the combined CP4 (C2 + P2) treatment on St2 but not in the C2 treatment on St2. Furthermore, there was an increase of released LDH in the CP1 (C1 + P1) on St2 but not in the C1 treatment on St2. These last results suggest a synergistic effect of the combined treatments CP4 and CP1 on the disruption of the cultures.

Regarding cheeses, the incorporation of the treated cells produced an effect only on the levels of lactose, AA and the levels of acetic acid. The lower levels of lactose in all the experimental cheeses in comparison with control ones were correlated with the increase of the β-gal activity in the treated cultures, described previously. In this way, several works have reported these treatments as a strategy to obtain low-lactose food products (Bury et al., 2001; Somkuti et al., 1998). Ethanol was efficient as permeabiliser and it is safe to use in food industry. For SDS, although it provided good levels of cell permeabilisation, its use in foods is very limited: it is only admitted in the United States and its application is related to a few foods such as egg whites (as emulsifier) or marshmallows (as whipping agent) (FDA, 2016). Nevertheless, Farag, Aly, and El-Alfy (1992) used SDS directly on the curd of blue cheese to increase the flavour. Additionally, the application of SDS directly on the starter to obtain an disrupted culture to accelerate cheese ripening was patented by Smith et al. (2003). Ultrasonic, heat shock and freeze shock treatments are safe methods to be applied in food. In this way, Lee et al. (2007) studied the heat-shock method on *Lb. helveticus* DPC 4571 for their use in enzyme-modified cheese production, and Sakakibara et al. (1994) reported an increase of the lactose hydrolysis when the fermentation was carried out under ultrasonic irradiation.

In the present work, we analysed two intracellular enzymes: LDH and β-gal, as indexes of the levels of cultures disruption. However, other intracellular enzymes could be released or to be more accessible due to the lysis or permeabilisation by the application of disruption treatments. In this sense, the marked increase of the level of the total and some individual AA in EU and ES cheeses due to the addition of treated cultures with ultrasound (M2) and SDS (C1) suggests the release of intracellular peptidases, which were active in the cheese matrix during ripening. In a similar way, Doolan and Wilkinson (2009) reported that the application of these both methods on *Lc. lactis* subsp. *cremoris* AM2, *Lc. lactis* subsp. *cremoris* HP and *Lc. lactis* subsp. *lactis* 303 resulted in highly permeabilised cells and enhanced accessibility to Pep X or Pep N.

The diversity of volatile compounds found in cheeses is characteristic of complex foods such as cheeses (Marilley & Casey, 2004). In general, the similar volatile compounds profile of control and experimental cheeses indicate that the disruption of the St2 strain by the studied treatments did not notably affect the release or accessibility of the enzymes involved in the production of these compounds.

It is known that amino acids act as precursors of several volatile compounds in cheeses throughout different pathways, catalysed by microbial enzymes (Ardö, 2006). However, in this work, the increase of amino acids did not correlate with an increase of volatile compounds, suggesting that *S. thermophilus* has not key enzymatic activities involved in the production of volatile compounds or they were inactive in the cheese matrix. Only acetic acid, which could derive from different substrates (such as lactose, glucose, amino acids, etc.) was found at higher amounts in some experimental cheeses. In contrast with our results about the impact of treated cells of *S. thermophilus*, disrupted cultures of several strains of lactobacilli have demonstrated significant changes in the ripening profiles of cheeses (Klein & Lortal, 1999).

5. Conclusions

In this work, we assessed the effect of eight disruption treatments and four combinations of them on the cell integrity of two strains of *S. thermophilus*, which are available in the market of dairy cultures. We identified enzymatic and mechanical treatments as the most effective for lysis, while chemical methods were effective as permeabilising agents. Physical methods were not suitable for the lysis or permeabilisation.

Our results direct us to select disruption treatments for these strains according to the intended use. Specifically, for food applications, ethanol or ultrasound treatments could be suitable for the production of lactose-hydrolysed dairy products or cheeses with higher proteolysis.

Although the use of mutanolysin, SDS and bead milling are not food grade methodologies, they provided information regarding the influence of the cell integrity on the metabolic pathways of *S. thermophilus* related to lactose fermentation, peptidolysis and flavour formation in cheeses.

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References

Aly, M. E. (1994). Flavour-enhancement of low-fat Kashkaval cheese using heat- or freeze-shocked *Lactobacillus delbrueckii* var. *helveticus* cultures. *Die Nahrung, 38*, 504–510.

Ardö, Y. (2006). Flavour formation by amino acid catabolism. *Biotechnology Advances, 24*, 238–242.

Bradley, R. L., Arnold, E., Barbano, D. M., Semerad, R. G., Smith, D. E., & Vines, B. K. (1993). Chemical and physical methods. In *Standard methods for the examination of dairy products* (pp. 433–531). Washington, DC, USA: American Public Health Association.

Bude-Ugarte, M., Guglielmotti, D., Giraffa, G., Reinheimer, J., & Hynes, E. (2006). Nonstarter lactobacilli isolated from soft and semihard Argentinean cheeses: Genetic characterization and resistance to biological barriers. *Journal of Food Protection, 69*, 2983–2991.

Bunthof, C. J., Van Schalkwijk, S., Meijer, W., Abee, T., & Hugenholtz, J. (2001). Fluorescent method for monitoring cheese starter permeabilization and lysis. *Applied and Environmental Microbiology, 67*, 4264–4271.

Burns, P. G., Patrignani, F., Tabanelli, G., Vinderola, G. C., Siroli, L., Reinheimer, J. A., et al. (2015). Potential of high pressure homogenization on probiotic Caciotta cheese quality and functionality. *Journal of Functional Foods, 13*, 126–136.

Bury, D., Jelen, P., & Kaláb, M. (2001). Disruption of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11,842 cells for lactose hydrolysis in dairy products: A comparison of sonication, high-pressure homogenization and bead milling. *Innovative Food Science & Emerging Technologies, 2*, 23–29.

Calasso, M., Mancini, L., Di Cagno, R., Cardinali, G., & Gobbetti, M. (2015). Microbial cell-free extracts as sources of enzyme activities to be used for enhancement flavor development of Ewe milk cheese. *Journal of Dairy Science, 98*, 5874–5889.

Chapot-Chartier, M.-P., Rul, F., Nardi, M., & Gripon, J.-C. (1994). Gene cloning and characterization of PepC, a cysteine aminopeptidase from *Streptococcus thermophilus*, with sequence similarity to the eucaryotic bleomycin hydrolase. *European Journal of Biochemistry, 224*, 497–506.

Coolbear, T., Pillidge, C. J., & Crow, V. L. (1994). The diversity of potential cheese ripening characteristics of lactic acid starter bacteria: 1. Resistance to cell lysis and levels and cellular distribution of proteinase activities. *International Dairy Journal, 4*, 697–721.

Cui, Y., Xu, T., Qu, X., Hu, T., Jiang, X., & Zhao, C. (2016). New insights into various production characteristics of *Streptococcus thermophilus* strains. *International Journal of Molecular Sciences, 17*, E1701.

Daryaei, H., Coventry, J., Versteeg, C., & Sherkat, F. (2010). Effects of high pressure treatment on glycolytic enzymes of *Lactococcus lactis* subsp. lactis, *Streptococcus thermophilus* and *Lactobacillus acidophilus*. *Innovative Food Science & Emerging Technologies, 11*, 245–249.

Doolan, I. A., & Wilkinson, M. G. (2009). Comparison of the effects of various attenuation methods on cell permeability and accessibility of intracellular enzymes in *Lactococcus lactis* strains. *International Dairy Journal, 19*, 215–221.

El Soda, M., Madkor, S., & Tong, P. S. (2000). Evaluation of commercial adjuncts for use in cheese ripening: 4. Comparison between attenuated and not attenuated lactobacilli. *Milchwissenschaft, 55*, 260–263.

Exterkate, F. A. (2006). Controlled permeabilization of *Lactococcus lactis* cells as a means to study and influence cheese ripening processes. *International Dairy Journal, 16*, 788–796.

Farag, A. A., Aly, M. E., & El-Alfy, M. B. (1992). Enhancement of blue cheese flavour using sodium dodecylsulphate and lipase. *Die Nahrung, 36*, 1–7.

FDA. (2016). *Code of Federal Regulations, Title 21 (Food and Drugs), Chapter 1 (Food and drug administration, department of health and human services), Part 172 (Food additives permitted for direct addition to food for human consumption)*. Silver Spring, MD, USA: US Food and Drug Administration.

FIL-IDF. (1982). *Formaggio e formaggio fuso. Determinazione della materia seca. Metodo di riferimento. N° 4:A*. Brussels, Belgium: International Dairy Federation.

FIL-IDF. (1993). *Latte. determinazione del tenore in azoto. Metodo di riferimento. N° 20:B*. Brussels, Belgium: International Dairy Federation.

FIL-IDF. (1997). *Lait et produits laitiers. Détermination de la teneur en matière grasse. Guide de directives générales appliqués aux methods butyrométriques. Norme FIL Internationale 152:A*. Brussels, Belgium: International Dairy Federation.

Geciova, J., Bury, D., & Jelen, P. (2002). Methods for disruption of microbial cells for potential use in the dairy industry—a review. *International Dairy Journal, 12*, 541–553.

Hannon, J. A., Wilkinson, M. G., Delahunty, C. M., Wallace, J. M., Morrissey, P. A., & Beresford, T. P. (2003). Use of autolytic starter systems to accelerate the ripening of Cheddar cheese. *International Dairy Journal, 13*, 313–323.

Hummel, W., & Kula, M.-R. (1989). Simple method for small-scale disruption of bacteria and yeasts. *Journal of Microbiological Methods, 9*, 201–209.

Husson-Kao, C., Mengaud, J., Cesselin, B., Van Sinderen, D., Benbadis, L., & Chapot-Chartier, M. P. (2000). The *Streptococcus thermophilus* autolytic phenotype results from a leaky prophage. *Applied and Environmental Microbiology, 66*, 558–565.

Joyce, E., Al-Hashimi, A., & Mason, T. J. (2011). Assessing the effect of different ultrasonic frequencies on bacterial viability using flow cytometry. *Journal of Applied Microbiology, 110*, 862–870.

Joyce, E., Phull, S. S., Lorimer, J. P., & Mason, T. J. (2003). The development and evaluation of ultrasound for the treatment of bacterial suspensions. A study of frequency, power and sonication time on cultured Bacillus species. *Ultrasonics Sonochemistry, 10*, 315–318.

Kebary, K. M. K., Salem, O. M., Hamed, A. I., & El-Sisi, A. S. (1997). Flavour enhancement of direct acidified Kareish cheese using attenuated lactic acid bacteria. *Food Research International, 30*, 265–272.

Klein, N., & Lortal, S. (1999). Attenuated starters: An efficient means to influence cheese ripening—a review. *International Dairy Journal, 9*, 751–762.

Kreft, M. E., & Jelen, P. (2000). Stability and activity of β-galactosidase in sonicated cultures of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842 as affected by temperature and ionic environments. *Journal of Food Science, 65*, 1364–1368.

Kreft, M. E., Roth, L., & Jelen, P. (2001). Lactose hydrolysing ability of sonicated cultures of *Lactobacillus delbrueckii* ssp. *bulgaricus* 11842. *Lait, 81*, 355–364.

Lee, B. H., Kilcawley, K. N., Hannon, J. A., Park, S. Y., Wilkinson, M. G., & Beresford, T. P. (2007). The use of viable and heat-shocked *Lactobacillus helveticus* DPC 4571 in enzyme-modified cheese production. *Food Biotechnology, 21*, 129–143.

López Morales, M. B., Bintsis, T., Alichanidis, E., Herian, K., Jelen, P., Hynes, E. R., et al. (2018). Soft cheeses (with rennet). In P. Papademas, & T. Bintsis (Eds.), *Global cheesemaking technology cheese quality and characteristics* (pp. 301–325). Chichester, UK: John Wiley & Sons, Ltd.

Madkor, S. A., Tong, P. S., & El Soda, M. (2000). Ripening of Cheddar cheese with added attenuated adjunct cultures of lactobacilli. *Journal of Dairy Science, 83*, 1684–1691.

Marilley, L., & Casey, M. G. (2004). Flavours of cheese products: Metabolic pathways, analytical tools and identification of producing strains. *International Journal of Food Microbiology, 90*, 139–159.

Middelberg, A. P. J. (1995). Process-scale disruption of microorganisms. *Biotechnology Advances, 13*, 491–551.

Milesi, M. M., McSweeney, P. L. H., & Hynes, E. R. (2008). Viability and contribution to proteolysis of an adjunct culture of *Lactobacillus plantarum* in two model cheese systems: Cheddar cheese-type and soft-cheese type. *Journal of Applied Microbiology, 105*, 884–892.

Milesi, M. M., Wolf, I. V., Bergamini, C. V., & Hynes, E. R. (2010). Two strains of nonstarter lactobacilli increased the production of flavor compounds in soft cheeses. *Journal of Dairy Science, 93*, 5020–5031.

O'Donnell, C. P., Tiwari, B. K., Bourke, P., & Cullen, P. J. (2010). Effect of ultrasonic processing on food enzymes of industrial importance. *Trends in Food Science & Technology, 21*, 358–367.

Peralta, G. H., Bergamini, C. V., Audero, G., Páez, R., Wolf, I. V., Perotti, M. C., et al. (2017). Spray-dried adjunct cultures of autochthonous non-starter lactic acid bacteria. *International Journal of Food Microbiology, 255*, 17–24.

Peralta, G. H., Bergamini, C. V., & Hynes, E. R. (2016). Aminotransferase and glutamate dehydrogenase activities in lactobacilli and streptococci. *Brazilian Journal of Microbiology, 47*, 741–748.

Sakakibara, M., Wang, D., Ikeda, K., & Suzuki, K. (1994). Effect of ultrasonic irradiation on production of fermented milk with *Lactobacillus delbrueckii*. *Ultrasonics Sonochemistry, 1*, S107–S110.

Smith, M. R., Browning, P. D., & Pawlett, D. (2003). *Cheese ripening process*. Patent US-6649200-B2.

Somkuti, G. A., Dominiecki, M. E., & Steinberg, D. H. (1996). Sensitivity of *Streptococcus thermophilus* to chemical permeabilization. *Current Microbiology, 32*, 101–105.

Somkuti, G. A., Dominiecki, M. E., & Steinberg, D. H. (1998). Permeabilization of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* with ethanol. *Current Microbiology, 36*, 202–206.

Somkuti, G. A., & Steinberg, D. H. (1994). Permeabilization of *Streptococcus thermophilus* and the expression of beta-galactosidase. *Enzyme and Microbial Technology, 16*, 573–576.

Tabanelli, G., Vernocchi, P., Patrignani, F., Del Chierico, F., Putignani, L., Vinderola, G., et al. (2015). Effects of sub-lethal high-pressure homogenization treatment on the outermost cellular structures and the volatile-molecule profiles of two strains of probiotic lactobacilli. *Frontiers in Microbiology, 6*, 1006.

Upadhyay, V. K., Huppertz, T., Kelly, A. L., & McSweeney, P. L. H. (2007). Use of high pressure treatment to attenuate starter bacteria for use as adjuncts for Cheddar cheese manufacture. *Innovative Food Science & Emerging Technologies, 8*, 485–492.

Upadhyay, V. K., & McSweeney, P. L. H. (2003). Acceleration of cheese ripening. In G. Smit (Ed.), *Dairy processing: Improving quality* (pp. 419–447). Cambridge, UK: Woodhead Publishing.

Vinderola, C. G., & Reinheimer, J. A. (2003). Lactic acid starter and probiotic bacteria: A comparative "in vitro" study of probiotic characteristics and biological barrier resistance. *Food Research International, 36*, 895–904.

Wang, D., & Sakakibara, M. (1997). Lactose hydrolysis and β-galactosidase activity in sonicated fermentation with *Lactobacillus* strains. *Ultrasonics Sonochemistry, 4*, 255–261.

Wilkinson, M. G., Guinee, T. P., & Fox, P. F. (1994). Factors which may influence the determination of autolysis of starter bacteria during cheddar cheese ripening. *International Dairy Journal, 4*, 141–160.

Yarlagadda, A. B., Wilkinson, M. G., O'Sullivan, M. G., & Kilcawley, K. N. (2014). Utilisation of microfluidisation to enhance enzymatic and metabolic potential of lactococcal strains as adjuncts in Gouda type cheese. *International Dairy Journal, 38*, 124–132.

Zotta, T., Ricciardi, A., Rossano, R., & Parente, E. (2008). Urease production by *Streptococcus thermophilus*. *Food Microbiology, 25*, 113–119.