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Review

Combined effect of pressure and temperature for yogurt production

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Abstract

Fermentation under non-conventional conditions has gained prominence in the last years, due to the possible process improvements. Fermentation under sub-lethal pressures is one of such cases, and may bring novel characteristics and features to fermentative processes and products. In this work, the effect of both pressure (10-100 MPa) and temperature (25-50 ºC) on yogurt production fermentation kinetics was studied, as a case-study. Product formation and substrate consumption were evaluated over fermentation time and the profiles were highly dependent on the fermentation conditions used. For instance, the increase of pressure slowed down yogurt fermentation, but fermentative profiles similar to atmospheric pressure (0.1 MPa) were obtained at 10 MPa at almost all temperatures tested. Regarding temperature, higher

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fermentative rates were achieved at 43 ºC for all pressures tested. Moreover, the inhibitory effect of pressure increased when temperature decreased, with complete inhibition of fermentation occurring at 50 MPa for 25-35 ºC, contrasting to 43 ºC where inhibition occurred only at 100 MPa. Therefore, an antagonistic effect seems to occur, since yogurt fermentation was slowed down by pressure increasing, on one hand, and by temperature decreasing, on the other hand. Additionally, some kinetic parameters were calculated and fermentation at 43 ºC presented the best results for yogurt production, with lower fermentation times and higher lactic acid productivities. Interestingly, fermentation at 10 MPa/43 ºC presented the optimal conditions, with improved yield and lactic acid production efficiency, when compared to fermentation at 0.1 MPa (efficiency of 75 % at 10 MPa, against 40 % at 0.1 MPa). As the authors are aware, this work gives the first insights about the simultaneous effect of pressure and temperature variation on a microbial fermentation process, which can be combined to modulate the metabolic activity of microorganisms during fermentation in order to improve the fermentative yields and productivities of the desired product.

Keywords

Fermentation, yogurt, lactic acid, high pressure, temperature, stress

1. INTRODUCTION

High Pressure (HP) is a commercial processing technology usually applied for non-thermal pasteurization of foods. However, the pressure effect on microorganisms depends on the pressure magnitude, with increasing pressure leading to a progressive inactivation of proteins and cell damage (Abe, 2007). Novel applications have been described for HP (Aertsen, Meersman, Hendrickx, Vogel, & Michiels, 2009; Mota,

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Lopes, Delgadillo, & Saraiva, 2013), some of them involving the use of sub-lethal levels of pressure as a mild stress condition to trigger general and specific stress responses by microorganisms (in a way to adapt and survive under these conditions). The activation of these stress responses usually involves changes in metabolic processes. This approach was also tested using some other emergent technologies (e.g., ultrasounds and electric fields). However, HP presented some advantages, namely in what regards to the possibility of applying the stress during the whole fermentation time without heating and the low energy requirements, reducing the process cost (Mota et al., 2018). Thus, the performance of fermentation under sub-lethal levels of pressure is an emergent concept, which may lead to changes in the fermentative rate and yield and/or shifts in the metabolic pathways with possible production of novel final products (Mota et al., 2013).

In fact, fermentative rate and yield of bioethanol production by *Saccharomyces cerevisiae* was enhanced by pressure levels of 5 and 10 MPa and the maximal ethanol production was obtained at 5 MPa (Picard, Daniel, Montagnac, & Oger, 2007). Regarding metabolic shifts under pressure, Bothun et al. (2004) observed a modification in product selectivity towards ethanol production rather than acetate by *Clostridium thermocellum* at 7.0 MPa and 17.3 MPa. In addition, bacterial cellulose produced by *Gluconacetobacter xylinus* under pressure was found to show profound morphological differences, when compared to the ones produced at atmospheric pressure (Kato et al., 2007). Another approach already tested is the application of HP-stresses only in the beginning of fermentation. Metabolic changes related to the production of lactic acid isomers by *Oenococcus oeni* were observed by Neto et al. (2016) when a stress of 100 MPa/8 h was applied. These differences suggest that sub-lethal levels of pressure may bring novel characteristics and features to both fermentative process and final product

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(Mota et al., 2013). Regarding food fermentations, HP was already applied during lactic acid fermentation for production of probiotic yogurt at 43 ºC, using commercial yogurt as inoculum (Mota, Lopes, Delgadillo, & Saraiva, 2015). In this case, fermentative rate was found to decrease with increasing pressure until total inhibition at 100 MPa, but the extension of fermentation time at 5 MPa allowed the production of yogurt with the characteristic pH (pH 4.5). Changes on milk caused by pressure during long treatments are not reported in literature, but alterations occurring in milk pressurized for short times have been studied, namely on whey proteins and micelles. β-lactoglobulin is the most sensitive protein to pressure (≥ 100 MPa, 25 ° C), while α-lactalbumin and bovine serum albumin can withstand up to 400 MPa, but higher temperatures result in higher protein denaturation. At 100 - 200 MPa there is a partial disintegration of the casein micelles and an apparent increase in the density of the remaining micelles, however, with increasing pressure (300 - 800 MPa) a decrease of size up to 50 % occurs (López-Fandiño, 2006).

In addition to pressure, temperature works as a thermodynamic variable, being widely used to investigate biological systems (Decaneto et al., 2015). Thus, the variation of fermentation temperature also has effects on fermentative processes and final products. For instance, in the case of yogurt production, the acidification rate and gel formation are highly affected by temperature (Lee & Lucey, 2004), with more viscous, smoother and slimy yogurts obtained when the temperature process is lowered from 43-45 ºC to 32-39ºC (Sodini, Remeuf, Haddad, & Corrieu, 2004).

Therefore, these two parameters (pressure and temperature) can be used together to modulate the fermentative processes, namely the metabolic activity of the microorganisms involved and possibly the characteristics of the final product. In fact, this approach was already applied to enzymatic systems, where acceleration of

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enzymatic reactions was obtained with the combination of pressure and temperature (Luong et al., 2016; Luong, Kapoor, & Winter, 2015; Ueda, Shinoda, & Kamaya, 1994; Van den Broeck, Ludikhuyze, Van Loey, & Hendrickx, 2000), due to enzymes stabilization against thermal inactivation by pressure (Aertsen et al., 2009; Czeslik, Luong, & Winter, 2017). However, this approach was never applied to fermentative processes, despite the possible entailed advantages. Therefore, the purpose of this work was to study the combined effect of pressure and temperature on fermentative processes, using yogurt production as a case-study. This dairy product was chosen because it corresponds to one of the most popular fermented product nowadays (Chilton, Burton, & Reid, 2015) and yogurt production is a relatively fast process, facilitating the experimental process for data generation. Therefore, the fermentation process was performed under different combinations of pressure (10-100 MPa) and temperature (25-50 ºC), in order to understand the effects on the acidification rate of starter cultures present in yogurt.

2. MATERIAL AND METHODS

2.1. Yogurt production

Milk preparation was performed based on Settachaimongkon et al. (2014) and Haque et al. (2001), with reconstitution of 10% (w v^{-1}) Nido whole milk powder (Nestlé, Portugal) in distilled water to obtain a final liquid milk with approximately 9.7% dry matter content. The prepared milk was pasteurized at 90 ºC for 20 minutes in a circulating water bath and it was then cooled rapidly to ambient temperature by immersion in running tap water. Thereafter, milk was stored overnight at 5 ºC.

Sample preparation consisted in the combination of the pasteurized milk with a commercial lactic acid lyophilized culture for yogurt production (Yo-Aktiv of ADMIX

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Ltd. composed by *Lactobacillus bulgaricus* and *Streptococcus thermophilus*) at a concentration of 2 g L^{-1}, accordingly to the manufacturer's instructions. After homogenization, the mixture was transferred to a heat sealed plastic bag resistant to high pressures.

The mixture was then incubated at different pressure and temperature conditions. The experiments were executed in two Hydrostatic presses (FPG13900 for room temperature experiments and FPG7100 for the remaining temperatures, both from Stanstead Fluid Power, Stanstead, United Kingdom) of our research group. While the FPG13900 equipment has three pressure vessels of 37 mm inner diameter and 520 mm height without temperature control, the FPG7100 equipment has a pressure vessel of 100 mm inner diameter and 250 mm height surrounded by an external jacket to control the temperature. In both equipments, a mixture of propylene glycol and water was used as pressurizing fluid.

Pressures of 10, 30, 50 and 100 MPa and temperatures of ≈25 (room temperature, RT), 35, 43 and 50 ºC were tested (Table 1), using fermentation under atmospheric pressure (0.1 MPa), and at the respective temperature, as control. During fermentation time, several samples were collected and stored at -20 ºC. Each experiment and analysis was performed in duplicate.

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2.2. Physicochemical analyses

2.2.1. Titratable acidity and pH

Acid production was monitored by determination of titratable acidity and pH. Titratable acidity was analyzed using a Titromatic 1S (Crison Instruments, S. A., Spain), accordingly to Chandan and Kilara (2013) with some modifications: 1.50 g of yogurt sample were diluted in 10.50 mL of water and then titrated with a 0.1N NaOH

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solution, until pH 8.9 was reached. The results obtained were expressed in % (w/w) of lactic acid. Additionally, pH of the fermentative medium was measured using a properly calibrated glass electrode (pH electrode 50 14, Crison Instruments, S. A., Spain), at 25 ºC.

2.2.2. Sugar concentration

For determination of sugar concentration, yogurt samples were first treated with Carrez I and Carrez II solutions to precipitate proteins and other high molecular weight molecules, but keep carbohydrates in solution (Fisher, Christison, Yang, Verma, & Lopez, 2014). Initially, 1.00 g of yogurt samples was added to 60 mL of distilled water and the suspension was incubated at 50 ºC for 15 minutes. Then, 2 mL of Carrez I solution [potassium hexacyanoferrate (II) ($K_4[Fe(CN)_6].3H_2O$)], 2 mL of Carrez II solution [zinc sulphate ($ZnSO_4.7H_2O$)] and 4 mL of a100 mM NaOH solution were added to the suspension. Finally, the mixture was diluted to a final volume of 100 mL with distilled water, mix thoroughly and the resulting solution was filtered through Whatman No. 1 filter paper (Megazyme, 2014).

Reducing sugars determination was measured according to the method described by Miller (1959), using 3,5-dinitrosalicylic acid (DNS) reagent. For that, 1.0 mL of the clear filtrate was added to 1.0 mL of DNS reagent and the mixture was incubated in a boiling water bath during 5 minutes. After cooling in an ice bath, the mixture was diluted with 10 mL of distilled water and the absorbance was measured at 540 nm. The concentration values were calculated using a calibration curve, obtained from glucose standard solutions, and were expressed in mg g^{-1} of yogurt. DNS reagent was prepared weighing 10 g of DNS and dissolving in 200 mL of a 2 N NaOH solution by heating with intensive stirring. Simultaneously, a solution of 300 g of potassium tartrate in 500

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mL of distilled water was prepared by heating with intense stirring. Both solutions were mixed and stirred and the final mixture was then diluted to 1 L with distilled water.

2.3.Organic acids and sugars determination

Extraction of organic acids and sugars of yogurt samples was performed following the method described by da Costa et al, (2016), with modifications. Briefly, 1.00 g of yogurt was homogenized with 5 mL of 45 mmol L^{-1} for 1 min in a vortex and the mixture was then stirred in an orbital shaker for 30 min at 240 rpm. The homogenates were centrifuged at 6000 rpm for 30 min at 4 ºC and the supernatants filtered through a 0.22 μm pore size membrane filter and stored at -20 ºC until HPLC analysis. The chromatographic system consisted in a HPLC Knauer system equipped with Knauer K-2301 RI and K-2501 UV detectors, and an Aminex HPX-87H cation exchange column (300 x 7.8 mm) (Bio-Rad Laboratories Pty Ltd, Hercules, CA, USA). The mobile phase used was 13 mM H_2SO_4, delivered at a flow rate of 0.6 $mL.min^{-1}$ and the column maintained at 65 °C. Peaks were identified by their retention times and quantified using calibration curves prepared with different standards.

2.4.Statistical analysis

The results obtained were tested at a 0.05 probability level and the combined effect of pressure and temperature was tested with a one-way analysis of variance (ANOVA), followed by a multiple comparisons test (Tukey HSD) to identify statistical significant differences between samples.

3. RESULTS AND DISCUSSION

3.1.Effect of pressure and temperature on yogurt fermentation

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Lactic acid fermentation was performed under several combinations of pressure and temperature. Initially, the effect of increasing pressure was studied only at 43 ºC, the optimal temperature of yogurt fermentation at atmospheric pressure (used in industry). Then, in order to observe the temperature influence on fermentation under pressure, a large spectrum of temperatures that can be used without inhibit the fermentation was tested (ranging from room temperature at ≈25 ºC (RT) to 50 ºC). Fermentation was monitored by pH variation (Figure 1), which is one of the most important physicochemical parameters in yogurt production, since the yogurt production process is considered finished when a pH of 4.5 is reached (corresponding to the isoelectric point for casein) (Hui, Nollet, Toldrá, Benjakul, & Paliyath, 2012). Thus, this value is represented by a dotted line in Figure 1, to easily identify the time needed for yogurt production in all cases studied.

In general, differences in the fermentative profiles were observed for each combination of pressure and temperature. Fermentation at atmospheric pressure was influenced by the temperature changes, decreasing the fermentative rate at RT, 35 and 50 ºC relatively to 43 ºC, and consequently increasing the time required to obtain yogurt. Thus, fermentation times were adapted for each set of temperature experiments (10 h for 43 ºC experiments; 24 h for 35 and 50 ºC experiments and 96 h for RT experiments). Lee and Lucey (2003) and Nguyen et al. (2014) also observed this decrease in the fermentative rate when an incubation temperature different from the optimal was used for yogurt production at atmospheric pressure. While slower enzymatic reactions and membrane solidification are behind the lower microbial growth rate when temperature decreases, structural cell components denaturation and enzyme inactivation are behind it when temperature is higher than optimal (FDA, 2003).

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Regarding the pressure influence, the increase of pressure was generally reflected in the decrease of pH variation rate. However, this effect was also dependent on the temperature applied in each case. For instance, fermentation at 10 MPa and 0.1 MPa showed similar profiles in almost all temperatures, except for 35 ºC, where the fermentative rate was slightly lower with a final pH slightly higher ($p < 0.05$). Increasing the pressure to 30 MPa slowed down fermentation at all temperatures, more considerably in some cases than in others. While at 43 and 50 ºC only a slight decrease was observed (with similar final pHs obtained at 43 ºC ($p > 0.05$), and close final pHs at 50 ºC, but significantly different ($p < 0.05$)),, a substantial decrease was observed at 35 ºC and RT ($p < 0.05$). In fact, yogurt typical pH was obtained for all fermentations at 10 and 30 MPa, with the exception to 30MPa/RT, within the longest fermentation time studied (96 h). However, the farther the fermentation conditions were from the conventional one (i.e., 0.1 MPa/43 ºC), the longer the fermentation times needed to produce yogurt. On the other hand, no substantial fermentation occurred when pressure increased to 50 and 100 MPa, with no pH variation at 50 MPa/RT, 50 MPa/35ºC and 100 MPa/43 ºC.

In summary, an increase of the pressure inhibitory effect was observed when temperature decreased, which was emphasized by the different pressures levels needed to inhibit fermentation at each temperature tested: 50 MPa at RT and 35 ºC, in contrast to 100 MPa at 43 ºC. Pressure increasing leads to inhibition of some cell processes and metabolic reactions essential for cell maintenance, depending on pressure resistance of the cell structure (Mota et al., 2018). For instance, cell membrane is one of the most pressure sensitive cellular components, among biological systems. As occurring at low temperatures, membrane fluidity decreases with pressure increasing, reducing the membrane permeability and consequently disrupting cell metabolism (Winter &

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Jeworrek, 2009). Thus, the combination of high pressures and low temperatures seems to compromise both cell structure and function to a higher extent than each non-optimal condition separately, since both have negative effects on cells.

On the other hand, microorganisms can withstand pressure due to the production of proteins that are able to protect cells against heat and pressure treatments (Abee & Wouters, 1999; Welch, Farewell, Neidhardt, & Bartlett, 1993). Thus, cells at high pressures and high temperatures are able to withstand these severe conditions more easily than cells at high pressures and low temperatures. In fact, higher temperatures cause an increment of membrane permeability (Chandler, 2017; Winter & Jeworrek, 2009), which compensates the opposite effect of high pressure effect on membranes. This can explain the fact that the increase of the inhibitory effect of pressure was not observed at 50 ºC, even though fermentation was longer than at 43 ºC. In fact, the fermentation profiles at 10 and 30 MPa were similar to the control fermentation at 50 ºC.

Comparing the results obtained at 43 ºC with Mota et al. (2015), fermentative rates also decrease with pressure increase, until no fermentation occur at 100 MPa. However, fermentation at 5 MPa already presented a decrease in the pH variation rate, in contrast to the present work, where fermentation at 0.1, 10 and 30 MPa presented similar profiles. These differences may be explained by the use of a different inoculum for yogurt production with different pressure resistance. In fact, commercial lyophilized starter cultures were used as inoculum in the present work, while commercial yogurt was used in Mota et al (2015).

3.2. Product formation and substrate consumption rates

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In addition to pH variation, titratable acidity and reducing sugars concentration were also monitored to give information about the product formation and substrate consumption, respectively. Then, the respective rates were calculated using the results correspondent to the exponential growth phase of the starter cultures.

Both fermentative rates (Figure 2) are in accordance to the pH variation results: i) increased as temperature increase up to 43 ºC, corresponding to an acceleration of fermentation; and ii) decreased, even if slightly, as temperature increase to 50 ºC, corresponding to a deceleration. Thus, the fastest yogurt production occurred at 43 ºC for all pressures tested (ranging from atmospheric pressure (0.1 MPa) to 50 MPa), which is usually reported as the optimal temperature for yogurt production at atmospheric pressure.

Regarding the pressure effect, fermentative rates mostly decreased with pressure increasing, with exception of fermentation at 50 ºC where the product formation rate (r_P, Figure 2a) was higher at 10 MPa and the substrate consumption rate (r_S, Figure 2b) was higher at 30 MPa. In this case, the higher r_P at 10 MPa may indicate that low pressures accelerate the lactic acid fermentation at 50 ºC, which do not occur at lower temperatures. A similar rate enhancement was already reported by Picard et al. (2007) for alcoholic fermentation by *S. cerevisiae* that was accelerated when fermentation occurred under pressure (5 and 10 MPa) with production of higher amounts of bioethanol. In fact, higher titratable acidities, expressed as % (w/w) lactic acid, were also achieved when lactic acid fermentation occurred at 10 MPa/50 ºC (data not showed). However, regarding r_S, the acceleration of substrate consumption at 10 MPa/50 ºC was not detected, with r_S similar to the one at 0.1 MPa. But, the highest r_S was obtained for the fermentation at 50ºC/30 MPa, which may be related with the need

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of more energy, i.e. more substrate, to withstand these harsh levels of pressure and temperature, in order to microbial cells survive, ferment and produce yogurt.

In summary, antagonistic effects on fermentation seem to occur when both pressure and temperature increase up to 43 ºC, since fermentation is accelerated by the temperature increase on one hand, and, on the other hand, is slowed down by the pressure increase. However, when temperature was increased to 50 ºC, this antagonistic effect was basically not verified, since fermentation was slowed down by temperature (and not accelerated, as expected) and, on the other hand, pressure did not slow down fermentation. Different stress responses could be behind this different behavior of cells towards pressure. As stated in the previous section, the production of heat-shock proteins caused by pressure increasing may help to withstand this higher temperature. In fact, the biosynthesis of proteins involved in the prevention of thermal degradation is already documented as one of the mechanisms of stress resistance to pressure for some lactobacilli strains (Bucka-Kolendo & Sokołowska, 2017).

3.3.Organic acids and sugars assessment

In order to deepen the study about the effect of pressure and temperature on yogurt fermentation, the presence of organic acids and sugars in the extracellular medium was evaluated by HPLC analysis. In this case, only fermentations at 10 and 30 MPa were studied, with fermentation at 0.1 MPa used as control. These cases were selected because yogurt was obtained within an experimental reasonable fermentation time, thus facilitating the experiments execution and data generation. The only exception was fermentation at 30 MPa/RT, where yogurt was not produced at the end of fermentation time but fermentation was almost complete.

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Lactose, galactose, glucose, lactic acid and citric acid were identified in all samples analyzed and their variation throughout fermentation is represented in Figure 3. However, since the concentration of citric acid remained approximately constant over the fermentation time, these results were not included in Figure 3. The presence of citric acid in the samples is explained by its presence on the milk used in this work. In fact, citric acid is the predominant organic acid in milk (Costa et al., 2015). Regarding the other compounds identified, lactose concentration had the tendency to decrease over fermentation time, while galactose and lactic acid increased and glucose remained constant during almost all fermentations tested.

Lactose is the major component of milk (with a concentration of 29.77 mg g^{-1} in the present work) and is the main substrate used by lactic acid bacteria during fermentation. Thus, as expected, lactose concentration decreased over time in all cases studied. However, different profiles were observed for each fermentation analyzed. While lactose concentration showed a linear decreasing pattern during fermentations at RT and 43 ºC, a marked decrease followed by a stabilization was observed at 35 and 50 ºC. In addition, pressure also affected lactose consumption, which was reflected in the different final concentrations obtained in each case. In general, higher final concentrations were achieved when fermentation occurred at higher pressures ($p < 0.05$), i.e. when fermentation was slower, indicating that lactose consumption was lower in these cases. However, some exceptions were observed. For instance, at RT, fermentation at 10 MPa presented a higher final concentration than fermentation at 0.1 and 30 MPa, which had similar final values ($p > 0.05$). This unexpected higher lactose consumption at 30 MPa may be related with the need of energy by the cells to trigger adaption mechanisms to the harsh conditions they were subjected to. In fact, while pH variation did not occur in the first 48 hours, approximately 20% of lactose was already

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consumed in this case. The other exception was observed at 50 ºC, where the fermentation at 0.1 MPa presented a higher final concentration than fermentation at 10 and 30 MPa. In this case, the final pH obtained was also slightly higher at 0.1 MPa than 10 and 30 MPa, which may explain this difference in lactose consumption.

In addition to lactose, galactose and glucose were also present in the samples. During fermentation, lactose is transported into the cell by permeases without any chemical modification, being afterwards hydrolyzed by β-galactosidase to glucose and galactose. Usually, glucose is catabolized via Embden–Meyerhof–Parnas (EMP) pathway, being galactose secreted from the cell (Tamime & Robinson, 1999). Thus, variation of galactose concentration during fermentation must be related with lactose variation, i.e. galactose concentration should increase when lactose concentration decreased. In fact, galactose concentration increased over time in all fermentations tested, with different variation profiles, as observed for lactose. In general, galactose concentration had a marked increase in the beginning of fermentation followed by a slight stabilization. The exceptions to this profile occurred when fermentation was slower (e.g., 10 MPa/RT, 30 MPa/RT, 30 MPa/50 ºC) with concentration increasing during all the fermentation time. Comparing with lactose variation, some differences were observed: i) at 35 ºC, the variation of lactose concentration at 30 MPa was slower than at the other pressures tested, while galactose variation was similar; ii) at 43 ºC, lactose was more consumed at 0.1 MPa, resulting in a lower final concentration ($p < 0.05$), but the final concentrations of galactose was similar for all pressures tested ($p > 0.05$); and iii) at 50 ºC, while lactose consumption was lower during the fermentation at 0.1 MPa, a lower increase of galactose concentration was observed at 30 MPa, resulting in lower final concentrations ($p < 0.05$). Therefore, these differences may indicate changes in lactose metabolism, due to the combined effect of pressure and temperature.

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In contrast to galactose, glucose is catabolized to pyruvate right after lactose hydrolysis and not expelled to the extracellular medium (Tamime & Robinson, 1999). Thus, it would be expected that glucose concentration in the extracellular medium would remain constant or even decrease during fermentation time. In fact, this behavior was observed in almost all fermentations tested, with the exception of fermentation at 30 MPa/RT and 30 MPa/35 ºC. In these cases, an increase of glucose content was observed in the beginning of fermentation, being followed by a decrease. Interestingly, these two conditions correspond to the fermentations with a lower fermentative rate (r_P, Figure 2a). One possible explanation may be that lactose hydrolysis and glucose catabolism are not affected by pressure to the same extent, resulting in an excess of glucose produced by lactose hydrolysis, when compared to the amount used to proceed the fermentative process. Thus, cells might expel this excess, increasing the glucose concentration in the extracellular medium. When the fermentation rate increased, more glucose is consumed, less is expelled and its concentration in the medium decreased. In fact, Neto et al. (2016) verified that enzymes can be more resistant to pressure than the microbial cell where they are present, since although pressure caused complete inactivation of *O. oeni*, malolactic enzyme maintained some residual activity. Therefore, pressure may have a similar effect on β-galactosidase and starter cultures of yogurt – *S. thermophilus* and *L. bulgaricus*.

With the results obtained for sugar content, the percentage of substrate consumption was calculated (Figure 4a). Taking into account that both galactose and glucose may be metabolized by the starter cultures (Hardie, 1986; Kandler & Weiss, 1986), substrate consumption was determined by mass balances. In most cases, about 30 % of the sugars present in milk were consumed by the starter cultures, with slight lower values obtained at higher pressures. However, some exceptions were observed: lower values were

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observed at 10 MPa/RT, 10MPa/43 ºC, 30 MPa/43 ºC and 0.1 MPa/50 ºC, and, on the other hand, a slightly higher value was observed for fermentation at 10 MPa/50 ºC. Interestingly, these differences correspond to lower and higher variations of lactose concentration, respectively. Thus, lactose variation is the predominant parameter in respect to substrate consumption during yogurt production.

Regarding acids produced during fermentation, lactic acid is the main product of carbohydrate metabolism of lactic acid fermentation. Yogurt bacteria usually perform homolactic fermentation, where only lactic acid is produced from pyruvate (Tamime & Robinson, 1999). Thus, lactic acid must be the main acid responsible for the acidity increase in yogurt samples. Analyzing the obtained results, lactic acid production was found to vary accordingly to the pH variation (Figure 1). For instance, the production was inhibited by increasing pressure at all temperatures tested, with similar final lactic acid concentrations obtained at 0.1 and 10 MPa (statically similar values were obtained at RT and 43 ºC ($p > 0.05$) and close but significantly different values were obtained at 35 and 50 ºC ($p < 0.05$)), but lower concentrations at 30 MPa ($p < 0.05$). In fact, during fermentation at 30 MPa/RT, lactic acid production only occurred between 48 and 96 hours of fermentation, due to the lowest fermentative rate observed. Interestingly, this effect was not observed in lactose concentration that decreased during the whole fermentation time, in contrast to glucose that reached the highest concentration after the 48 hours of fermentation. Therefore, these results support the explanation given above, suggesting that lactose hydrolysis and lactic acid production were not affected by pressure to the same extent.

Pressure inhibition of lactic acid production was reflected by lower productivities (Q_P) at 30 MPa for each temperature tested (Figure 4b). Fermentations at 43 ºC presented higher Q_P values, which was expected since lower fermentative times were

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needed to obtain yogurt at 43 ºC. Thus, similar values were obtained at 35 and 50 ºC (24 hours of fermentation in both cases) and lower ones at RT (96 hours of fermentation).

In order to relate lactic acid production to the sugar consumption, two kinetic parameters were calculated – fermentation yield and efficiency (Figures 4c and 4d, respectively). While fermentation yield gives information about the amount of lactic acid produced per sugar consumed, fermentation efficiency is the percentage of lactic acid that was actually produced relatively to the amount that could be theoretically produced with the sugars consumed during the process (da Fonseca, 2007). Analyzing the results obtained for both parameters, similar profiles were observed, i.e. higher yields correspond to higher efficiencies. However, a standard profile for pressure influence was not clearly identified. Generally, pressure increasing seemed to decrease fermentation yield and efficiency, i.e. sugars were consumed but lactic acid was not produced to the same extent under pressure, which may suggest that sugars were used by bacteria to other cellular processes (such as, adaptation mechanisms to pressure), but not for lactic acid production. However, some exceptions were observed at 10 MPa/RT, 10 MPa/43 ºC and 30 MPa/43 ºC, which presented higher values of yield and efficiency than the respective control samples (at atmospheric pressure). In these cases, bacteria were able to produce high concentrations of lactic acid with less sugars consumed, indicating that sugar catabolism towards lactic acid production was improved at these conditions.

Interestingly, analyzing the temperature influence at each pressure, different profiles were observed when fermentation was performed under pressure, compared to atmospheric pressure. While yield and efficiency increased with temperature increasing up to 50 ºC at 0.1 MPa, under pressure, higher values were observed at 43 ºC. In fact, values higher than 0.1 MPa were observed under pressure, with fermentation

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efficiencies of 75.09 % and 69.89 % at 10 and 30 MPa, respectively, against 39.63 % at 0.1 MPa. Improved fermentative yields at 10 MPa were also reported by Picard et al. (2007) during alcoholic fermentation. The authors assumed that this increased activity under pressure might be related with the enhancement of glucose uptake, glycolysis and/or fermentation pathways, which can also explain the results obtained here.

Therefore, this work provided the first results about the combined effect of pressure and temperature on microbial fermentation, applied to yogurt production. All the results presented in this work pointed that the most suitable conditions for yogurt fermentation were, in fact, at 43 ºC, where lower fermentation times were required to produce yogurt and higher lactic acid productivities were achieved. However, the optimal conditions observed were 10 MPa/43 ºC, being even better than fermentation at 0.1 MPa. Thus, fermentation under sub-lethal levels of pressure can bring relevant improvements to the fermentative process, namely lower sugars consumption, higher productivity, yield and efficiency, when compared to fermentation at atmospheric pressure. These changes may indicate changes in the metabolic activity of microorganisms under pressure, with the metabolic pathway of lactic acid production being stimulated, while other pathways were reduced, increasing lactic acid productivity, yield and efficiency, as a consequence.

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4. CONCLUSIONS

This work gives the first insights on the combined effect of pressure and temperature on a microbial fermentation process and kinetics. Simultaneous variation of both pressure and temperature influenced the fermentative rates, with the pressure effect being dependent on incubation temperature. In general, higher pressures and lower

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temperatures slowed down yogurt production, with fermentations at 43 ºC presenting the highest fermentative rates. Using kinetic parameters to characterize the influence of both variables on the fermentative process, interesting differences in the processes fermented under pressure were achieved. Improved yields were observed for fermentations under pressure (10 and 30 MPa) at 43 ºC, which were reflected into lactic acid efficiencies of 70-75 %, in contrast to 40 % at atmospheric pressure. Thus, the fermentative process showed modifications under pressure, with microorganisms more effectively converting lactose into lactic acid. Therefore, pressure and temperature may be used as process variables to modulate the metabolic activity of microorganisms during fermentation and improve the productivities and yields of the desired product. Since these modifications may be converted in a final product with different properties, the yogurt produced under pressure should be analyzed regarding its microbiological, rheological, sensorial and nutritional properties, in order to describe the pressure influence on the final product of fermentation.

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Figure 1. pH variation during fermentation at room temperature (≈25 ºC) (a), 35 ºC (b), 43 ºC (c) and 50 ºC (d), under different conditions of pressure: 10 MPa (diamonds), 30 MPa (triangles), 50 MPa (stars) and 100 MPa (crosses). Control fermentations at 0.1 MPa are represented as squares.

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Figure 2. Product formation rate (a) and substrate consumption rate (b) correspondent to fermentation at room temperature (≈25 ºC), 35 ºC, 43 ºC and 50 ºC, under different conditions of pressure: 0.1 MPa, 10 MPa, 30 MPa and 50 MPa.

Figure 3. Lactose, galactose, glucose and lactic acid concentrations during fermentation at room temperature (≈25 ºC), 35 ºC, 43 ºC and 50 ºC, under different conditions of pressure: 10 MPa (diamonds) and 30 MPa (triangles). Control fermentations at 0.1 MPa are represented as squares.

Figure 4. Consumed sugars (a), lactic acid productivity (b), lactic acid on sugars yield (c) and lactic acid efficiency (d) correspondent to fermentation at room temperature (≈25 ºC), 35 ºC, 43 ºC and 50 ºC, under different conditions of pressure: 0.1 MPa, 10 MPa and 30 MPa.

Table 1. Fermentation conditions (temperature, pressure and time) of each fermentative process tested during this work.

Temperature	Pressure	Time
RT (≈25 ºC)	0.1 MPa	96 h
	10 MPa	
	30 MPa	
	50 MPa	
35 ºC	0.1 MPa	24 h
	10 MPa	
	30 MPa	
	50 MPa	
43 ºC	0.1 MPa	10 h
	10 MPa	
	30 MPa	
	50 MPa	
50 ºC	0.1 MPa	24 h
	10 MPa	
	30 MPa	

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	50 MPa	

Highlights

- Simultaneous variation of pressure and temperature affected the fermentative rate.
- Pressure effect depends on incubation temperature.
- Higher pressures and lower temperatures slowed down yogurt production.
- Fermentation at 10MPa/43ºC improved fermentative yield and lactic acid efficiency.
- Pressure and temperature may be used to modulate microorganisms metabolism.

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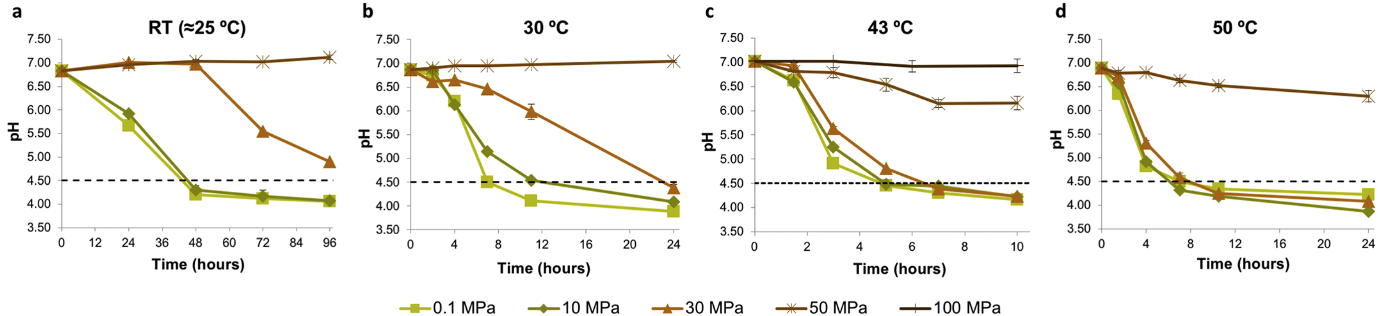


Figure 1

a

2.000
1.600
1.200
0.800
0.400
0.000

r_P (mg g^{-1} h^{-1})

RT (≈25 ºC)
35 ºC
43 ºC
50 ºC

b

2.000
1.600
1.200
0.800
0.400
0.000

r_S (mg g^{-1} h^{-1})

RT (≈25 ºC)
35 ºC
43 ºC
50 ºC

0.1 MPa
10 MPa
30 MPa
50 MPa

Figure 2

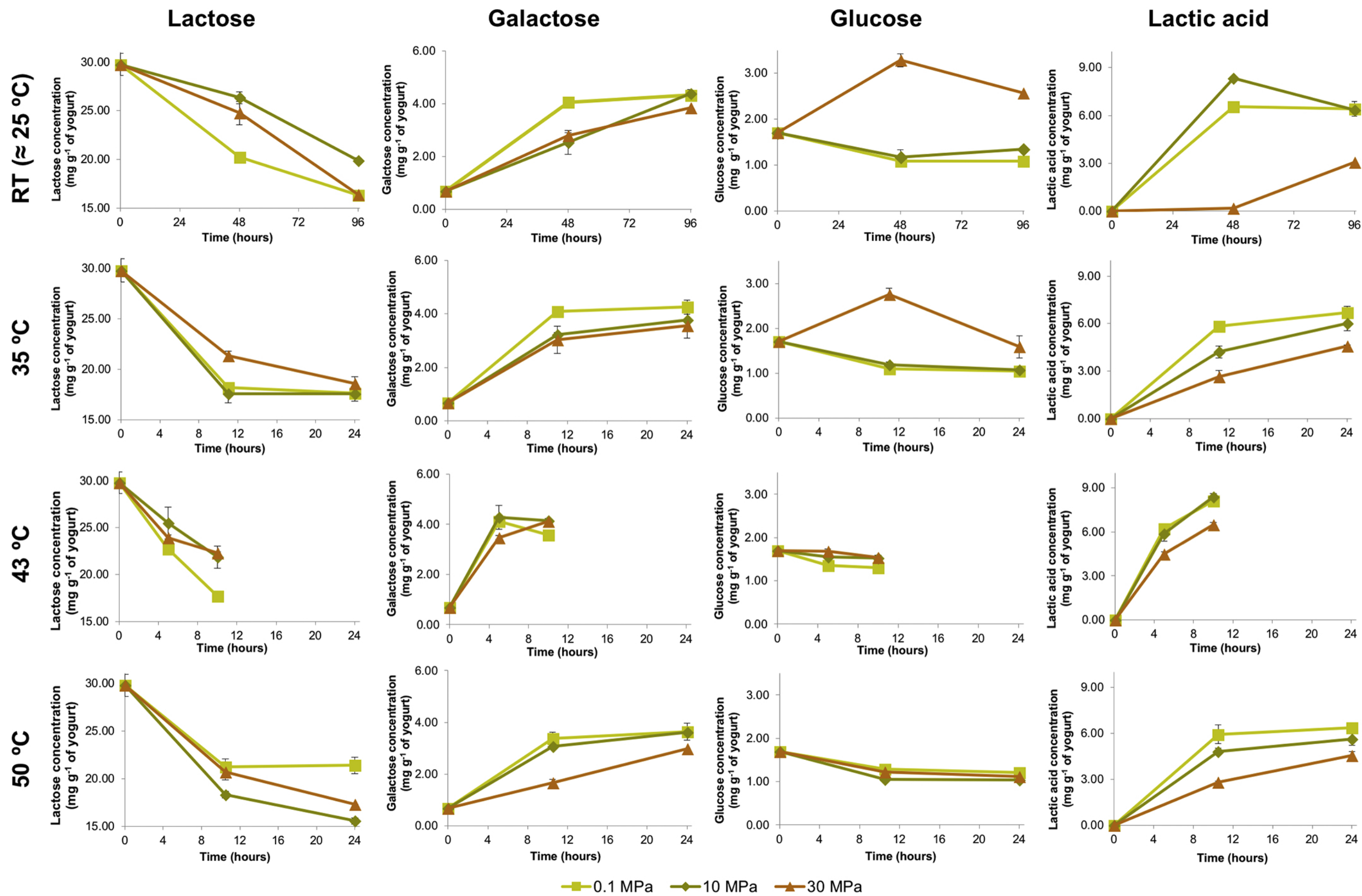
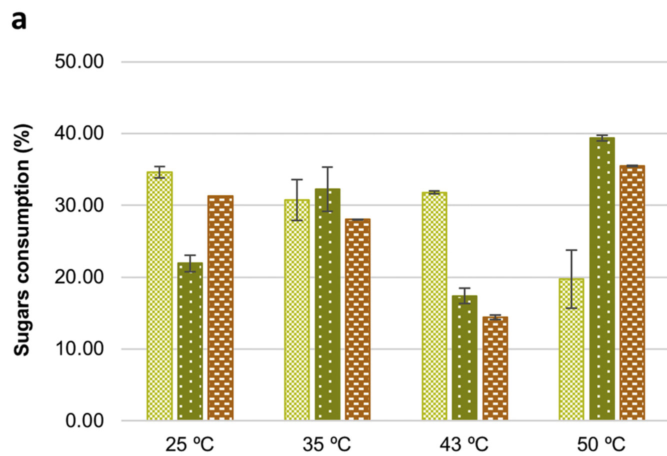
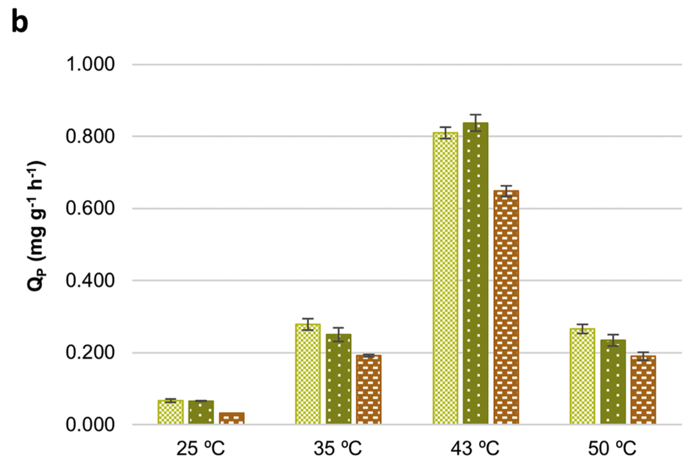
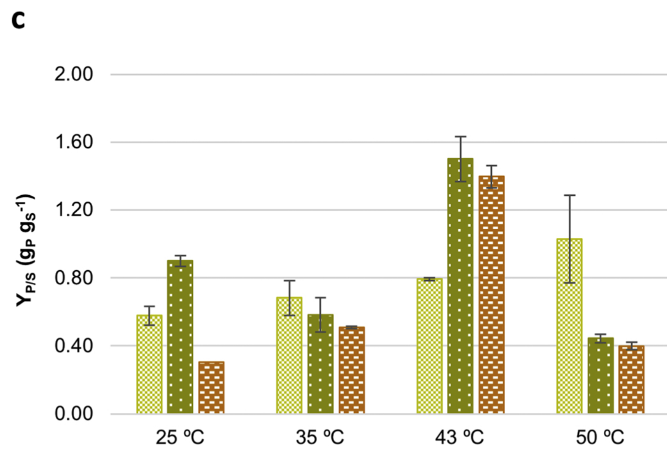
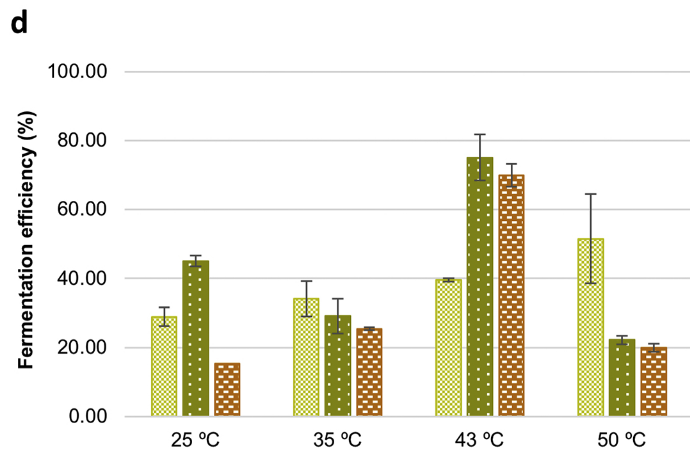


Figure 3









0.1 MPa 10 MPa 30 MPa

Figure 4