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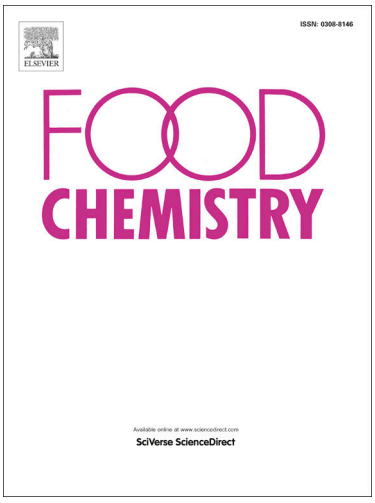

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A chemical study of yoghurt produced under isostatic pressure during storage

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Abstract

Yoghurt fermented under sub-lethal high pressure (10, 20, 30 and 40 MPa at 43 ºC), and afterward placed under refrigeration (4 ºC for 23 days) was studied and compared with yoghurt fermented at atmospheric pressure (0.1 MPa). For a deeper analysis, a metabolite fingerprinting by nuclear magnetic resonance (NMR), sugars and organic acids assessment by high performance liquid chromatography (HPLC), total fatty acids (TFA) determination and quantification by gas chromatography with a flame ionization detector (GC-FID) were performed. Metabolomic analyses revealed that only 2,3-butanediol, acetoin, diacetyl and formate vary with the increase of pressure and probable relation with pressure influenced diacetyl reductase, acetoin reductase and acetolactate decarboxylase. Yoghurts fermented at 40 MPa had the lowest content in lactose (39.7% of total sugar reduction) and the less content in TFA (56.1%). Further research is of interest to understand more about fermentation processes under sub-lethal high pressure.

Keywords: Fermentation under pressure; Yoghurt; Total fatty acids; Sugars and organic acids;

1. Introduction

Yoghurt is a semi-solid fermented milk product and is defined by the Food and Drug Administration (FDA) as a fermented dairy product derived from the fermentation of milk by two species of bacterial cultures, *Streptococcus thermophilus* (*S. thermophilus*) and *Lactobacillus delbrueckii ssp. bulgaricus* (*L. bulgaricus*), commonly named as lactic acid bacteria (LAB) (Freitas, 2017).

LAB do not possess the cytochrome system for electron transport or enzymes to operate the anaplerotic pathways and tricarboxylic acid cycle, the energy can only be supplied by the fermentation of carbohydrates (sugars) (Sharma et al., 2021).

In the homofermentative pathway, LAB convert glucose into lactic acid via the Embden-Meyerhof-Parnas (EMP) pathway. This process generates two molecules of lactic acid for every molecule of glucose consumed, leading to a high yield of lactic acid, while by homofermentation only lactic acid is produced as end product. Therefore, homofermentative LAB used in yoghurt production only produce lactic acid as their main end product. Differently, heterofermentative LAB can use various substrates other than glucose as a carbon source, such as fructose or pentoses, through the phosphoketolase pathway (PKP). This pathway produces not only lactic acid but also ethanol, acetic acid, and CO_2 as metabolic end products. Heterofermentative LAB have a lower yield of lactic acid than homofermentative LAB, but they can produce various flavor and aroma compounds that contribute to the taste and aroma of yoghurt (Chen et al., 2017).

In both systems, glucose and galactose converge at dihydroxyacetone phosphate and glyceraldehyde-3-phosphate, where the three-carbon sugars become further oxidised to phosphorylated by phosphoenolpyruvate (PEP) and then pyruvate kinase produces pyruvate, which is converted into lactic acid by lactate dehydrogenase (LDH).

The enzymatic activity, namely lipolytic, in homogenised milk is higher than in non-homogenised milk due to the destruction of the protective layer of fat globule, where lipases are placed, and released (Tamime & Robinson, 2007), which can result in distinct yoghurt. For example, fermentation of full fat milk with *S. thermophilus*, *L. bulgaricus* or *L. acidophilus* resulted in different effects on milk lipids, and, according to Sharma et al. (2021), there is a significant increase in saturated fatty acids (SFA) and oleic acid (C18:1 c9) and a decrease in linoleic (C18:2 c9, c12) and linolenic (C18:3 c9, c12, c15) acids in the glyceride fraction. Thus, the increase of free fatty acids (FFA) was moderate, nevertheless, the monoglyceride fraction disappeared completely upon fermentation and the changes in cholesterol content are not significant (Tamime and Robinson, 1999). During the manufacture and along yoghurt storage, a appreciable increase of volatile fatty acids (VFA) occurs, but this increase depends on several variables, such as the strains of the starter bacteria, type of milk, duration and temperature of incubation, processing conditions (thermal pasteurization) of the milk and/or the age of yoghurt (Murgia et al., 2019; Sharma & Ramanathan, 2021).

Yoghurts' popularity as food largely depends on its sensory characteristics, with aroma and taste being the most important. Yoghurt is widely appreciated for its delicate and low intense acidic flavour (Aryana & Olson, 2017). So, flavour is an important factor determining food product acceptability and preference for consumers (Cheng, 2010). These compounds may be divided into four main categories: Non-volatile acids (e.g. lactic, pyruvic, oxalic, and succinic); Volatile acids (e.g. acetic, propionic and butyric);

Carbonyl compounds (e.g. acetaldehyde, acetone, acetoin and diacetyl); Miscellaneous compounds (e.g. certain amino acids and compounds derived from protein, fat and lactose degradation) (Tamime and Robinson, 1999).

The study of dairy products' fermentation under sub-lethal isostatic pressure has increased in the last years (Lopes et al., 2020; Lopes, Mota, Pinto, et al., 2019; Lopes, Mota, Sousa, et al., 2019; Mota et al., 2015; Ribeiro et al., 2020). It is known that pressure influences negatively the fermentation rate: with the increase of pressure there is a gradual inhibition of fermentation until stops at pressures about 100 MPa (Lopes, 2013). However, information concerning the characteristics of these yoghurts is very scarce, with the available literature covering and focusing the physical and chemical parameters (Lopes, 2018; Vieira et al., 2019). So, the aim of this study is evaluating the characteristics (sugars, organic acids and total fatty acids, TFA) and understand how LAB alter their performance and products when the fermentation process takes place under sub-lethal isostatic pressure (10-40 MPa, 43 °C). This work is a continuation of the study of refrigeration storage (4 °C for 23 days) of yoghurts produced under sub-lethal high pressure (10, 20, 30 and 40 MPa at 43 ºC) in comparison with the fermentation process at atmospheric pressure (0.1 MPa) (Vieira et al., 2019). Briefly, in the aforementioned study, there were reported higher colour variations for yoghurts fermented under pressure, yet not perceived by naked eye, right after the fermentation process was finished and during the shelf-life evaluation studies, no major pH variations were observed, and the yoghurt firmness increased by increasing the yoghurt fermentation pressure.

2. Material and Methods

2.1. Yoghurt preparation

Yoghurt was produced according to the instructions provided by the inoculum manufacturer (Iogurte Caseiro Condi 28 g, Condi, Camarate, Portugal). One sachet of 7 g of inoculum was added to 1 litter of commercial pasteurized whole milk (Vigor, Lactogal Produtos Alimentares S.A, Porto, Portugal) that was purchased at a local supermarket. The mixture was well homogenised and then was fractioned in small (5 x 4 cm, containing 10 mL in two divisors) and medium (8 x 10 cm, containing 80 mL) polyamide/polyethylene bags (IdeiaPack – Comércio de Embalagens, LDA, Bodiosa, Viseu, Portugal) with 90 μm of thickness. The bags were stored at 4 ºC before fermentation for 24 h.

2.2. Yoghurt fermentation and storage

Fermentation was carried out under different hydrostatic pressures set at 0.1, 10, 20, 30 and 40 MPa, all performed at 43 °C, which is the optimal temperature of the LAB for yoghurt production (Tamime & Robinson, 2007). The pH was measured with a properly calibrated pH meter for semi-solid food (Testo 205 pH, Barcelona, Spain) during the fermentation process, and the fermentation process was ended when pH value reached 4.5.

The fermentations under high pressure were performed in a lab-scale high pressure equipment (Stansted Fluid Power FPG7100 FoodLab, Stansted, United Kingdom), using a mixture of propyleneglycol:water (40:60 v/v) as pressurization fluid, for samples fermented under 10 to 40 MPa. The HP equipment used has a pressure vessel of 2 L, and can be operated up to 900 MPa, from -20 to 110 °C. Samples fermented under atmospheric pressure (0.1 MPa) were immersed in a water bath during the fermentation period. The pH was periodically measured throughout the fermentation (with measurements being carried with 30 minutes interval as the pH approached 4.5) until a pH value of 4.5 was reached. To measure the pH the pressure vessel was decompressed and recompressed within 2 minutes time (this procedure was found to have no effect on fermentation time in previous tests (Lopes, 2018).

2.3. Metabolomics analysis by nuclear magnetic resonance (NMR)

One and half millilitres of yoghurt were transferred to an eppendorf (2 mL), centrifuged (at 8000 g for 15 minutes, at room temperature) (Centrifuge-mixer CM-50M, ELMI Ltd., Riga, Latvia) and then filtered (white and plain membrane filter of cellulose acetate; 0.22 µm (25 mm), Advantec - Japan). The supernatant (1 mL) was then dried in a vacuum centrifuge for about 24 h. Before NMR spectral acquisition, the samples were reconstituted using 600 µL of phosphate buffer (100 mM, pH 3.0) containing 0.01 % (wt/wt) of 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid, sodium salt (TSP-d4) as a chemical shift and intensity reference. The mixture was then transferred into 5 mm NMR tubes to be analysed.

^{1}H NMR spectra were recorded at 300 K in a Bruker Avance DRX 500 spectrometer (Bruker BioSpin, Germany), operating at a proton frequency of 500.13 MHz, equipped with an actively shielded gradient unit with a maximum gradient strength output of 53.5 Gcm-1 in a 5 mm inverse probe. For each sample, a 1D 1H NMR spectrum was acquired using the noesypr1d pulse sequence (Bruker pulse program library) with water presaturation. For all spectra, 128 transients were collected into 32,768 (32 K) data points with a spectral width of 10000 Hz, an acquisition time of 3.3 s and relaxation delay of 5 s. Each free induction decay was zero-filled to 64 k points and multiplied by a 0.3 Hz exponential line-broadening function prior to Fourier transformation. TopSpin 3.2 software was used to manually phase, and baseline correct the spectra. The spectra were exported as a matrix, by Amix-Viewer, using R-Studio in-house scripts and subsequently normalised to TSP. The spectra were overlaid and checked in iNMR to see whether alignment was required. If required, the speaq, rolps, BiocInstaller, ChemoSpec, classyfire, gdata, ggplot2, gplots, MassSpecWavelet, matrixStats, mclust, muma, pheatmap, plyr, R.utils, RColorBrewer, reshape2, seqinr and zoo packages was used in R software. To align all peaks the baselineThresh used was 2000, signal-to-noise ratio (SNR) Thresh was 40 and the maxshift used was 80 for all spectra, except for water zone.

2.3.1. Multivariate data analysis

The multivariate analysis was applied to the aligned spectra, using the ropls package (Thévenot et al., 2015) in R software. Differences among sample groups were identified using by Pareto scaled data followed by principal component analysis (PCA). The

identification of relevant metabolites was carried out by comparing the spectra with those of standard compounds from the Biological Magnetic Resonance Data Bank, the Human Metabolome Database, FooDB and the Chenomx NMR Suite software. The relative amounts of the NMR metabolites and the effect size were determined by integrating the area under the most well-separated metabolite peak using in-house R scripts. Pairwise t-tests were carried out using the False Discovery Rate (FDR) to adjust for multiple testing. Effect sizes were calculated and corrected for small sample sizes.

2.4. Organic acid and sugar assessment by high performance liquid chromatography (HPLC)

Triplicate samples of yoghurt, taken at the 1st and 23rd days of storage, were assayed for glycolysis. One gram was added to 5 mL of 13 mmol L−1 sulfuric acid (H_2SO_4) and vortexed for 1 min. The mixture was then stirred in an orbital shaker (VWR® Incubating Orbital Shaker, Model 3500I) for 30 min at 240 rpm at room temperature following another 1 min in vortex. The mixture was then centrifuged (Heraeus Biofuge Stratos centrifuge, Thermo Electron corporation, Waltham, Massachusetts, United States) at 6,000 rpm for 30 min at 4 °C and the supernatants were filtered through a 0.22 μm pore size membrane filter (white and plain membrane filter of cellulose acetate; 0.22 μm (25 mm), Advantec - Japan) and stored at -20 °C until analysis by HPLC. The HPLC system was composed of an ion exchange Aminex HPX-87H column (300 × 7.8 mm) (Bio-Rad) maintained at 40 °C and a Knauer K-2301 RI (refractive index) detector. The mobile phase used was 13 mmol L−1 sulphuric acid, delivered at a rate of 0.6 mL min−1. The running time was 30 min and the injection volume were 30 μL (Lopes, Mota, Sousa, et al., 2019).

Peaks were identified by their retention times and quantified using standard curves prepared with the mix of the different standards (lactose, glucose and galactose for sugars and lactic, citric, and formic acids for organic acids).

2.5. TFA determination and quantification by gas chromatography with a flame ionization detector (GC-FID)

As the authors are aware, this is the first time that a yoghurt fermented under pressure is characterized according to its FA profile. For the analysis of the fatty acids (FA) profile in yoghurt, triplicate samples of yoghurt, taken at 1 and 23 days of storage, were transmethylated to obtain the methyl esters of FA (FAME). About 700 mg of yoghurt were transferred to glass tubes and 200 μL of tritridecanoin (internal standard; C13) (1.7 $mg.mL^{-1}$) were added. Then, 800 μL of hexane, 2.25 mL of methanol (MeOH) and 240 μL of sodium methoxide (5.4 M) were also added, and the mixture was homogenised by vortexing and heated at 80 °C for 10 min. The tubes were cooled in ice, and 1.25 mL of N,N-dimethylformamide and 1.25 mL of H_2SO_4/MeOH (3 M) were added, vortexed and heated at 60 °C for 30 min. The mixture was again cooled in ice, and 1 mL of hexane was added, homogenised by vortexing for 30 s and centrifuged for 5 min at 1250 *g* at 18 °C. The upper layer of the resulting solution was collected for further GC-FID analysis.

The GC-FID used in FAME analysis was composed of a gas chromatograph HP6890A (Hewlett-Packard, Avondale, Pennsylvania, USA), a flame-ionization detector (GC-FID) and a BPX70 capillary column (60 m × 0.25 mm × 0.25 μm; SGE Europe Ltd, Courtaboeuf, France). Hydrogen was used as the carrier gas at 20.5 psi, the injector temperature was 250 °C, the injection volume was 1 μL (25:1 split) and the FID detector temperature was 275 °C. The oven temperature program was as follows: 60 °C (held 5 min), then raised at 15 °C/min to 165 °C (held 1 min) and finally at 2 °C/min to 225 °C (held 2 min). For the individual identification of fatty acids, Supelco 37 and FAME from CRM-164 were used. Also, calculation of response factors and detection and quantification limits (LOD: 0.79 μg FA/mL; LOQ: 2.64 μg FA/mL) were assayed with GLC-Nestlé36 protocol, as used by *Universidade Católica do Porto - Escola Superior de Biotecnologia.*

Fatty acids were quantified through the correlation of the area of the internal standard with the corresponding concentration, and assuming the same response for each individual fatty acid.

2.5.1. Nutritional (lipidic) quality indices

There are several indices to be used as indicators for determining whether a diet is atherogenic or promotes coronary heart diseases (CHDs) (Chalabi et al., 2018). Based on the FA composition, the atherogenicity and thrombogenicity indices were calculated. The index of atherogenicity (IA) was calculated using **Equation 1** that indicates the relationship between C12, C14, and C16 (pro-atherogenic factor) and unsaturated FA (USFA), as performed by (Chalabi et al., 2018; Naydenova et al., 2014; Senso et al., 2007; Ulbricht & Southgate, 1991)

$$IA = \frac{C12 + (4 \times C14) + C16}{\sum MUFA + PUFA_{n-6}{}^{*} + PUFA_{n-3}{}^{*}} \qquad \textbf{Equation 1}$$

*n-6 and n-3 are, respectively, FA omega-6 and omega-3, MUFA (monounsaturated fatty acids), PUFA (polyunsaturated fatty acids)

The ratio of C14, C16, and C18 (pro-thrombogenetic) to USFAs (anti-thrombogenetic) is described as the index of thrombogenicity (IT). This index refers to the tendency for clot formation in the blood vessels. The IT value was calculated according to **Equation 2**:

$$IT = \frac{C14 + C16 + C18}{(0.5 \times \sum MUFA + 0.5 \times PUFA_{n-6} + 3 \times PUFA_{n-3}) + \frac{PUFA_{n-3}}{PUFA_{n-6}}} \qquad \textbf{Equation 2}$$

*n-6 and n-3 are, respectively, FA omega-6 and omega-3, MUFA (monounsaturated fatty acids), PUFA (polyunsaturated fatty acids)

Other indicators included the ratio of omega-6/omega-3, monounsaturated fatty acids (MUFA)/ polyunsaturated fatty acids (PUFA), and the PUFA to SFA ratios were also calculated.

2.6. Statistical analysis

The results obtained were statistically analysed using two-way Analysis of Variance (ANOVA), followed by the Tukey's Honestly Significant Differences test, at a significance of 5 %, to infer statistical differences/similarities between conditions and storage days. For this, it was defined that different upper-case letters in tables and figures indicate statistically significant different ($p < 0.05$) values for a given day of storage at different fermentation pressures, while lower-case letters indicate statistically significant different ($p < 0.05$) values for different days of cold storage at a fermentative pressure. All the performed analyses were done in triplicate and all these values were counted for the statistics on pressure variation and storage day.

3. Results

3.1. Metabolomics analysis by NMR

NMR spectra are very difficult to analyse and sometimes it is difficult to separate the different peaks, as sugars – namely lactose, glucose and galactose – because they have peaks in common, however the principal peaks are identified and described in **Table 1**. The sugar peaks are the sum of galactose, lactose and glucose content/signal, and were divided into nine sub-groups.

In order to identify some of the metabolites present in the yoghurt samples, spectral comparisons with databases were performed. Regarding the full spectra of the different yoghurts, no obvious differences could be seen. The peaks with higher intensity corresponded to lactate and sugars, namely lactose and galactose. Minor compounds could also be observed in the aromatic (5.8 – 9.0 ppm) and aliphatic (0.5 – 3.1 ppm) regions. In these cases, the differences observed between samples were not as pronounced as for the aromatic region, but different intensities were obtained for peaks identified as 2.3-butanediol, acetate, acetoin, diacetyl and for unknown_2.

In order to identify the differences observed for samples fermented under different pressure conditions, a PCA was carried out using a dataset generated from the full ^{1}H NMR spectra. PCA is an unsupervised statistical analysis that is widely used as a first exploratory step in metabolomics studies. This statistical tool converts high dimensional data into fewer dimensions, maintaining as much variance from the original data as possible (Boccard et al., 2010; Nyamundanda et al., 2010). The PCA model showed a good fit ($R2X = 0.74$), with the first and second principal components (PC1 (t1) and PC2 (t2)) explaining 48 and 14 % of the total variance, respectively. The PCA scores plot revealed no significant and clear separation between the control samples (fermented under 0.1 MPa), samples subjected to pressure (fermented under 10, 20, 30 and 40 MPa) and sample storage time (1, 7, 15 and 23) (**Figure 1**).

In order to do a semi-quantitatively to compare the compositional changes between the yoghurt samples analysed, the normalized areas of the compounds were identified and calculated. Firstly, the identification of the signals corresponding to the metabolites present in the yoghurt samples was performed. The identification of different sugars was impossible due to the overlap of several signals in the sugar region, however other important yoghurt components were successfully identified, such as lactate, citrate, formate, pyruvate, diacetyl, acetoin, acetaldehyde, acetate, alanine, and 2,3-butanediol. Several unknown metabolite peaks were also observed. As mentioned previously, in addition to lactate production, starter cultures can also produce several compounds in lower amounts that are responsible for yoghurt flavour. In these cases, pyruvate is used as a metabolic precursor of the mixed acid metabolism. By analysis of the spectra, signals corresponding to some of these compounds were identified, including pyruvate, acetate, formate, acetaldehyde, diacetyl, acetoin and 2,3-butanediol.

No statistical differences ($p < 0.05$) were verified between the content of each compound (namely acetaldehyde, acetate, diacetyl, lactate, alanine, sugars, pyruvate and the unknown compounds) along yoghurt storage, except for 2,3-butanediol that increases between the 7th and 15th day of storage for yoghurt fermented under 40 MPa. Generally, there were no statistical differences ($p < 0.05$) between the content of compounds in yoghurts fermented under different pressures, as seen for acetaldehyde, acetate, lactate, alanine, pyruvate and sugars, except for 2,3-butanediol, acetoin, diacetyl and formate.

The compounds that contribute to the taste and aroma of yoghurt varied in terms of relative abundance between the samples. Acetoin showed different abundances between the yoghurt fermented under 40 MPa and the control (fermented under 0.1 MPa). On the other hand, in all analysed days, acetoin was more abundant in the yoghurts fermented under 20, 30 and 40 MPa, but it was observed a difference between acetoin and diacetyl and formate, the last ones are more abundant in the control yoghurt samples.

The abundance of 2,3-butanediol compound is lower in the control sample for the first day of storage when compared with the other samples. However, its content seems to increase on the 7th day of storage and is then stabilizes until the 15th day for all samples, except for the fermented under 40 MPa that increase their 2,3-butanediol content.

As mentioned before, both diacetyl and acetoin are important for the typical yoghurt aroma, being responsible for the butter-like flavour. The production of these two compounds is linked, since acetoin is the reduced form of diacetyl, produced with the irreversible action of diacetyl reductase (Cheng, 2010). Therefore, the fermentation conditions used during this work may have affected the activity of diacetyl reductase, when higher pressures cause an activity increase, due to the higher acetoin levels observed in the samples fermented with higher pressure. The same conclusion can be applied to acetoin reductase that reduce acetoin in to 2,3-butanediol. In the other hand the abundance of acetaldehyde is similar for all samples, which may suggest that the enzyme diacetyl synthase is not affected (positively) by pressure, so diacetyl and acetoin are formed by α-acetolactate (derived from pyruvate) and, possibly, pressure also active acetolactate decarboxylase.

The results obtained by the analysing spectra from 1D ^{1}H NMR was a pertinent approach to understand how different the matrix of the different yoghurts is. The principal compounds were sugars and lactose, and the biggest differences between the yoghurts were in the abundance of the flavour compounds. In parallel, it was possible to verify a

possible increase in the activity of some enzymes, such as acetoin reductase, diacetyl reductase, acetolactate decarboxylase and acetolactate synthase, but more studies are needed to confirm these expectations. On the other hand, β-gal, diacetyl synthase and lactate dehydrogenase possibly are not affected by pressure.

3.2. Organic acids and sugar content

Lactose, glucose, galactose, lactic and citric acids were identified in all analysed samples, namely at the 1st and 23rd days of storage. The compounds were identified by their retention time (min), namely lactose (7.39), citric acid (8.26), glucose (8.69), galactose (9.39) and lactic acid (12.91).

Lactose is one of the major constituents of milk, and the primary substrate consumed by LAB during fermentation, which produces lactic acid by metabolizing glucose and galactose. Since galactose is metabolized after glucose into lactic acid, it is anticipated that lactose will decrease and lactic acid will increase during fermentation, along with a decrease in glucose concentration relative to galactose concentration, as previously described in the introduction section. The results of this analysis are consistent with these expectations, as depicted in **Figure 2**. Comparing the control sample with samples fermented under 20 and 30 MPa, the lactose content decreases considerably ($p < 0.05$) with increasing pressure (**Figure 2-A**). Between the 1st and 23rd day of yoghurt storage, there were no statistically significant differences ($p > 0.05$) in lactose content. During cold storage, β-gal continues to convert lactose into glucose and galactose (reducing sugars).

In addition to lactose, galactose and glucose were also identified in the samples. During fermentation, lactose is hydrolysed by β-gal to glucose and galactose, to be transported into the cell by permeases without chemical modification (Tamime & Robinson, 1999). Thus, variation of galactose concentration during fermentation may be related with lactose variation, i.e., galactose concentration should increase when lactose concentration decreased.

The values obtained for glucose content are very different for the different yoghurts, as represented in **Figure 2-B**. The LOQ for glucose was 0.01 mg/g of yoghurt and the samples fermented under 0.1 MPa (1st and 23rd day) and 20 MPa (only for 1st day) had glucose content lower than the LOQ. Yoghurts fermented under 10 and 20 MPa had a significant increase ($p < 0.05$) of glucose during storage, which means that there was lactose metabolization by LAB during storage. However, the content in glucose did not exceed 1.5 mg/g of yoghurt for any sample. On the other hand, for yoghurts fermented under 10, 30 and 40 MPa, in the first day of storage, some glucose was detected, which can indicate a slower fermentation rate. For yoghurts fermented under 30 and 40 MPa, glucose content variation during storage was not significant ($p > 0.05$).

In case of the other monosaccharide, galactose, its content was about 2 to 7-fold higher than glucose for the different samples and there was much higher content on the 1st day of storage, as represented in **Figure 2-C**. There were no significant differences ($p > 0.05$) between storage periods, except for the yoghurt samples fermented under 20 and 30 MPa, wherein an increase was observed for glucose at 20 MPa. These results show that fermentation was ongoing, and lactose continued to be metabolized as well as other

minor sugars, by enzymes that can be activated by pressure. On the other hand, a bigger difference ($p < 0.05$) was observed between the yoghurts fermented under 0.1 and 10 MPa and the others, as these yoghurts had higher galactose content. This happens since galactose is not metabolized by the microorganisms of the yoghurt starter, releasing this monosaccharide to the yoghurt matrix.

Lactic acid that is produced in the fermentation of lactose contributes to the sour taste of yoghurt by decreasing pH and grants the characteristic texture. Lactic acid content was similar to the citric acid, as represented in **Figure 2-D**. The yoghurt fermented under 0.1 MPa, for the 1st day of storage, presented the highest average value of lactic acid (7.893 ± 0.836 mg/g of yoghurt), however, this value is only statistically different ($p < 0.05$) from the samples fermented under 20 and 30 MPa, which had the lower content (5.209 ± 0.153 and 5.908 ± 0.051 mg/g of yoghurt, respectively). During storage there were no significant variations ($p > 0.05$), except for the yoghurt fermented under 20 and 30 MPa, for which there was an increase ($p < 0.05$) in lactic acid content was observed. These values are in accordance with the previously discussed, as lactose seems to be reduced throughout the storage. Even though glucose and galactose increased during storage, lactic acid also increased, which means that lactose was metabolized into glucose and galactose that contribute to the increase of lactic acid.

Citric acid is a natural preservative present in milk, and an antioxidant. It is known that its content decreases with the age of milk (Supplee & Bellis, 1921), however, this content does not influence the rate of fermentation unless it is added after milk pasteurization (reduce 13.4 % of fermentation time) (Schmidt, 2009). In this case, the citric acid content in milk was not accessed. However, the fermentation of milk for each condition was performed in 4 consecutive days and the milk packages belonged to the same lot (batch). As such, the initial content of citric acid was expected to be similar in all milk packages. If this is correct, it means that pressure could have influenced the final content of this acid in yoghurt, as represented in **Figure 2-E**. In all samples, except for those fermented at 20 MPa, citric acid content did not vary ($p > 0.05$) along storage. However, in all of them, except for the control sample (0.1 MPa) an increase of the average value in the 23rd day was observed. The yoghurt fermented under 20 MPa had the lower citric acid content in the first day (5.392 ± 0.172 mg/g of yoghurt) and the fermented under 0.1 MPa had the higher content for the same day (9.134 ± 1.81 mg/g of yoghurt). These results mean that the yoghurts fermented under pressure have less citric acid content.

In general, β-gal seems to be more active when yoghurts are fermented under pressure, since lactose content at the first day of storage was lower, but more studies are needed. β-gal also remains, probably, active during storage (increase of the glucose and galactose contents) and the fermentation of lactose still slowly occurs, what can be explained by the presence of LAB and justifies the decrease of pH (Vieira et al., 2019).

The whole fresh milk used in this work had 4.8 g of sugars/ 100 mL of milk (48 mg/g), namely lactose, which means that the lactose in the control sample (yoghurt fermented under 0.1 MPa) was reduced by about 22.1 %. However, the input of pressure increases lactose metabolization: 10 MPa reduced 26.4 % of lactose, 20 MPa reduced 41.4 %, 30 MPa reduced 43.3 % and 40 MPa reduced 39.7 %.

On the other hand, the whole fresh milk used was probably rich in citric acid and is the reason why the final content in yoghurt of this acid was very similar to the lactic acid

content, so, both contribute to the pH decrease. However, the samples which were fermented under higher pressure had lower citric acid content, which suggests a catabolism of this compound during fermentation or storage, since the bacteria used cannot metabolize this acid. To sum up, the mean proportions of lactose:glucose:galactose in relation to the total sugars were similar in all yoghurts in the first day of storage, approximately 17:0:3. However, the same did not occur on the 23rd day where the mean proportions varied with pressure (0.1, 10, 20, 30 and 40 MPa), namely 16:0:3, 15:1:4; 16:1:4; 19:0:4; 15:0:3, respectively. This means that LAB undergo different changes during fermentation and their enzymes, namely β-gal, will act differently throughout the storage. On the other hand, the mean proportions of lactose:lactate were similar in each yoghurt and in the days of storage, being about 4:1.

Lopes et al., (2019) also investigated the variation of carbohydrates and organic acids in yoghurts fermented at 43 ºC under various pressures (0.1, 10, and 30 MPa). For this, the milk was reconstituted with milk powder and contained 29.77 mg lactose/g. Although the initial percentage of lactose was different, the results can be compared based on the lactose reduction, or the amount of unmetabolized lactose in the yoghurt. Contrary to what was observed in this study, those authors observed a greater lactose reduction in the control yoghurts than in those fermented under pressure (10 and 30 MPa), for which they observed comparable reduction proportions. The glucose and galactose contents of all samples were comparable (1.50 and 4.00 mg/g, respectively), which contradicts our findings. Similar amounts of lactic acid were found in both manuscripts, but citric acid was not identified in one. These differences may be the result of the matrix and LAB mixture used.

An informal sensorial analysis made at the laboratory revealed that, despite of not being observed major pH changes in yoghurts fermented under pressure, these were perceived as less acidic when compared to those fermented at atmospheric pressure, being indeed an interesting topic for future research.

3.3. TFA profile

In the fermentation process, LAB change the milk composition, such as fatty acid profiles, which can differ from one product to another. For this reason, in this work were analysed all FA, mainly the free FA and the conjugated/ esterified FA to triacylglycerols, diacylglycerols, monoacylglycerols and phospholipids to understand how different the matrix of the yoghurts fermented under pressure were.

According to the number of carbon atoms and dietary safety, the identified FA were divided into three main groups: short-chain FA (SCFAs) (C4, C6, C8 and C10), SFAs (C12, C14, C15, C16, C17, C18, C20, C22 and C24), and USFAs including MUFAs (C10:1 t2, C12:1, C14:1 c9, C15:1, C16:1 c7, C16:1 c9, C17:1 c10, C18:1 t12, C18:1 c9, C18:1 t15 and C18:1 c11) and PUFAs (C18:2 c9, c12 (*n*-6), C18:3 c9, c12, c15 (*n*-3), C18:9 c9, t11 (CLA) and C20:4 c5, c8, c11, c14). Moreover, there were identified some isomers (i) and anti-isomers (ai) of some FA (C13i, C13ai, C14i, C17i, C17ai). The compounds were identified by their retention time (**Table 1 – Supplementary tables**) comparing with other yoghurt spectra.

In all samples it was possible to identify and quantify thirty-three FA, whose content was higher than the LOQ. Our results showed that the FA profiles and their content of a sample fermented under each pressure does not change significantly ($p > 0.05$) along refrigerated storage. However, the yoghurts fermented under different pressures had different FA content in both storage days studied.

The milk used had 3.6 g of fat/100 mL of milk and 2.4 g of that are SFA. In terms of TFA, the yoghurt fermented under atmospheric pressure presented higher content 28006.5 ± 2547.1 μg/mg of yoghurt (1st day of storage) and with the increase of the applied pressure the content in TFA decrease 5.4, 14.6, 53.0 and 56.1 % for yoghurts fermented under 10, 20, 30 and 40 MPa respectively. This decrease is also noted in some groups of FA (SCFA, SFA and MUFA) and the more noticeable differences are between the yoghurts fermented under low pressures (0.1 and 10 MPa) and the fermented under higher pressures (20, 30 and 40 MPa) ($p < 0.05$). These results suggest that FA might be being used by LAB (to take energy or to adapt their membranes to assure pressure resistance, as it will be explained below) or being led to the formation of volatile compounds. The most interesting case is the yoghurt fermented under 10 MPa that had higher content in PUFA but also in *n*-3 and *n*-6 FA for the first day of storage.

The relative quantity of FA found in yoghurts can be seen in Table 2. Despite a decrease in TFA content, the percentage of each FA group does not remain constant relative to its TFA content. This indicates that each fatty acid may be affected differentially (either by an increase or a decrease in concentration) when the fermentation pressure is increased. In fact, it appears that increasing the fermentation pressure increases the relative proportion of total saturated fatty acids (SCFA + SFA) in fermented yoghurts under pressure, whereas the proportions of MUFA and total FA n-6 tend to decrease as the pressure rises. Higher proportions of PUFA, total n-3 FA, and trans-FA are found in yoghurts fermented at 10, 20, and 30 MPa. The FA content of the yoghurt fermented at atmospheric pressure is according with some authors (Chalabi et al., 2018; Güler & Gürsoy-Balcı, 2011; Júnior et al., 2012)**.** However, there are others studies concerning the effects of high pressure on fatty acids, however, just were noted changes when are applied higher pressures in meat (>350 MPa, during 20 min at 20 °C) (He et al., 2012), other study concluded that pressure (700 MPa) induces some conformational changes at the hydrocarbon skeleton on USFA in solid samples, while the liquid ones remain unchanged (Povedano et al., 2014), even though the results cannot be compared, as this work aimed a different range of pressures (10–40 MPa) during a long period of time at higher temperatures (43 °C).

The membrane of LAB can be modified due to pressure applied and to perform physiological functions in hostile environments, bacteria potentially remodel the membrane by changing the ratio of **(i)** saturation to unsaturation, **(ii)** *cis* to *trans* unsaturation, **(iii)** branched to unbranched structure, and **(iv)** acyl chain length. FA containing single or more unsaturated bonds have more bulky conformation than their saturated counterparts do, thus allowing higher conformational freedom and lesser packing of the membrane (Abe, 2015).

Natural cell membranes are a complex mixture of phospholipids, sterols, and numerous membrane proteins. Therefore, it is difficult to provide a straightforward account of the effect of high pressure on the phase behaviour of the membranes, their structure, activity of membrane proteins, and cell growth and viability (Abe, 2015). However, Beal et al., (2001) studied the FA composition of the cell membrane of *S.*

thermophilus and their change by alteration of some factors: incorporating oleic acid in the culture medium, fermentation pH, addition of glycerol as cryoprotective agent and duration of storage (at -20ºC). Firstly, there were identified nine FA in the cell membrane of *S. thermophilus*, namely C14:0, C16:0, C16:1, C18:1 c9, C18:1 c11, C19:1, C20:0, C20:1, the same that were identified by other authors in *L. bulgaricus* except C20:1. When the culture media was incorporated with oleic acid (C18:1 c9), the content in SFA decrease (C14:0, C16:0, C18:0, C20:0) but the content in C18:1 c9, C19:1 and C20:1 increase, so the ratio of USFA/SFA increased. The same was noticed when the fermentation pH decreased to 5.5. These results suggest that FA incorporated in milk must be integrated into LAB cell membrane due to the content in oleic acid, pH diminishing and pressure, although there is no evidence of this latter factor. Our results are in accordance with the results obtained by these authors, as represented in **Table 2** the MUFA content (% of TFA) decrease, mainly C18:1 c9, and the SFA content increase (% of TFA) that means that the SFA of LAB cell membrane are replaced by MUFA to increase their pressure resistance.

Nevertheless, it is known the importance of fat in the perception of food, and to modify the physical properties of food, including mouthfeel, appearance and structure. Fat is also important as a flavour precursor, flavour carrier and flavour release modulator, for these reasons it is very important a volatile compounds study to understand if these FA are possibility used as its precursors.

3.3.1. Lipid quality parameters

Dietary FA components such as SFA are associated with an increased risk of cardiovascular diseases, CHDs and mortality (Chalabi et al., 2018). Is recommend a limiting SFA intake and replacing them with PUFAs and MUFAs according to some epidemiological studies and clinical trials (Siri-Tarino et al., 2010). (Chalabi et al., 2018) cited that dietary SFA (C12 to C18) are indicators of atherogenic/ thrombogenic disorders whereas MUFAs, especially oleic acid, and some PUFAs such as linoleic (*n*-6) and a-linolenic acid (*n*-3), and the ratio of PUFAs to SFAs are indicators for a diet that will promote CHD. PUFAs are very susceptible to peroxidation, thereby contributing to CHDs, so, PUFA-rich diets should be consumed cautiously. Therefore, n-6 PUFA to n-3 PUFA, PUFA to SFA and MUFA to PUFA ratios could be considered as important parameters by which to determine the nutritional value of a food (Butler et al., 2011). The aim of this FA study is to compare the FA composition and related lipid quality of yoghurt fermented under pressure and the conventional one (fermented under 0.1 MPa).

In parallel to the quantification of TFA, we also studied some parameters/index to understand the nutritional quality of each yoghurt. IA, IT and the ratio of omega-6/omega-3, MUFA/PUFA, and the PUFA/SFA were calculated (**Table 3**). IA and IT index were very similar for all yoghurts along the storage. It is perceptible that both atherogenic and thrombogenic indices are very low, which can be attributed to the higher content in USFA comparing to C12, C14, C16 and C18. Note that C14, C16 and C18 are associated with high serum cholesterol and low density lipoprotein (LDL) cholesterol levels as risk factors for CHD and C18 is a thrombogenic SFAs, which accelerates blood clotting and the formation of platelet aggregation (Briggs et al., 2017; Müller et al., 2003).

Moreover, these results revealed that the *n*-6/*n*-3 and MUFA/PUFA ratios are higher for yoghurts fermented under 0.1 MPa and lower for the fermented under 20 and 30 MPa, that is probably a good result, as FA *n*-3 should prevail, because all intermediates of lipid metabolism from linoleic acid (*n*-6) are more harmful, for example prostaglandins and leukotrienes that are thrombogenic agents. Also, an excessive intake of PUFAs exerts undesirable effects such as oxidative stress induction and the *n*-6/*n*-3 ratio (as an index) is used in the prognosis of heart disease, diabetes and obesity, and many studies recommend that this ratio should be below 4 - a ratio of 4/1 was associated with a 70% decrease in total mortality (Simopoulos, 2008, 2016). On the other hand, the MUFAs are as effective as PUFAs in lowering serum cholesterol and the MUFA/PUFA ratio of a diet can be used as an indicator for protection from heart diseases (Naydenova et al., 2014). On the other hand, our findings indicate that the PUFA/SFA ratios in the control and for all samples fermented under pressure were lower than 0.4, which is in accordance with the recommendations made by the World Health Organization (WHO) (World Health Organization, 2003).

The lipid content of yoghurt is mostly derived from the milk used to produce it, but LAB can also contribute to lipid breakdown and modification. Enzymes produced by LAB can hydrolyse triglycerides into free fatty acids, which can change the fatty acid profiles of yoghurts. As a result, this can in fact lead to changes in the texture, flavour, and aroma of the yoghurt. Likewise, some strains of LAB can also produce some short-chain fatty acids such as lactic and acetic acid, which contribute to the tangy flavour of yoghurt (Chen et al., 2017).

4. Conclusion

This work examined sugars, organic acids, and total fatty acids in yoghurt fermented under pressure and stored at 4°C for 23 days. According to NMR metabolomics, 2,3-butanediol, acetoin, diacetyl, and formate vary with pressure increase, indicating that some enzymes may be affected by pressure: diacetyl reductase, acetoin reductase, and acetolactate decarboxylase may increase, while β-gal, diacetyl synthase, and lactate dehydrogenase may not and further research on the impact of pressures on enzymes during fermentation is needed.

For control samples (yoghurt fermented at 0.1 MPa), lactose consumption was decreased by 22.1 %, whereas those fermented at 40 MPa were lowered by 39.7 %. However, the mean proportions of lactose/lactic acid were similar for each yoghurt and for the days of storage, about 4:1, indicating that pressure-fermented yoghurts convert lactose to lactic acid at the same proportion as observed at atmospheric pressure. For yoghurts fermented under 10, 30, and 40 MPa on the first day of storage, lactose, glucose, and galactose vary in relation to total sugars, while control samples showed a higher fermentation rate during refrigerated storage. Galactose is at significantly greater amount than glucose, yet it declines with pressure but does not change during storage, due to the fact that LAB does not completely metabolize galactose, releasing it into the yoghurt matrix.

TFA content decreased with pressure, but the relative fraction of FA groups did not. Fermented yoghurts under pressure had more total saturated FA (SCFA + SFA) but less MUFA and total FA n-6. Yoghurt fermented under 10, 20, and 30 MPa had greater PUFA, total FA n-3, and trans FA percentages. LAB cell membranes may replace SFA with MUFA to boost pressure resistance, which may

explain the findings, although FA can also be used by LAB as a carbon source or to make more volatile chemicals. These yoghurts have decreased sugar and fat content and high lipid quality indices, making them a beneficial choice for consumers. Although pressure-fermented yoghurts ferment slower they may have distinct organoleptic and functional features and may be healthier than atmospheric-pressure-fermented ones. However, more research is needed to understand LAB behaviour under pressure and its health advantages.

Conflict of Interest

The authors have no conflict of interest to declare.

CRediT authorship contribution statement

Patrícia Vieira: Investigation, Writing – Original Draft, Writing – Review and Editing. **Carlos A. Pinto:** Investigation, Validation, Writing – Original Draft, Writing – Review and Editing.

Brian James Goodfellow: Validation, Writing – Review and Editing.

Ana M. Gomes: Supervision, Writing – Review and Editing.

Sérgio Sousa: Investigation, Validation.

Manuela Machado: Investigation, Writing – Original Draft.

Ivonne Delgadillo: Supervision, Writing – Review and Editing.

Jorge A. Saraiva: Resources, Supervision, Writing – Review and Editing.

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762 Figures:

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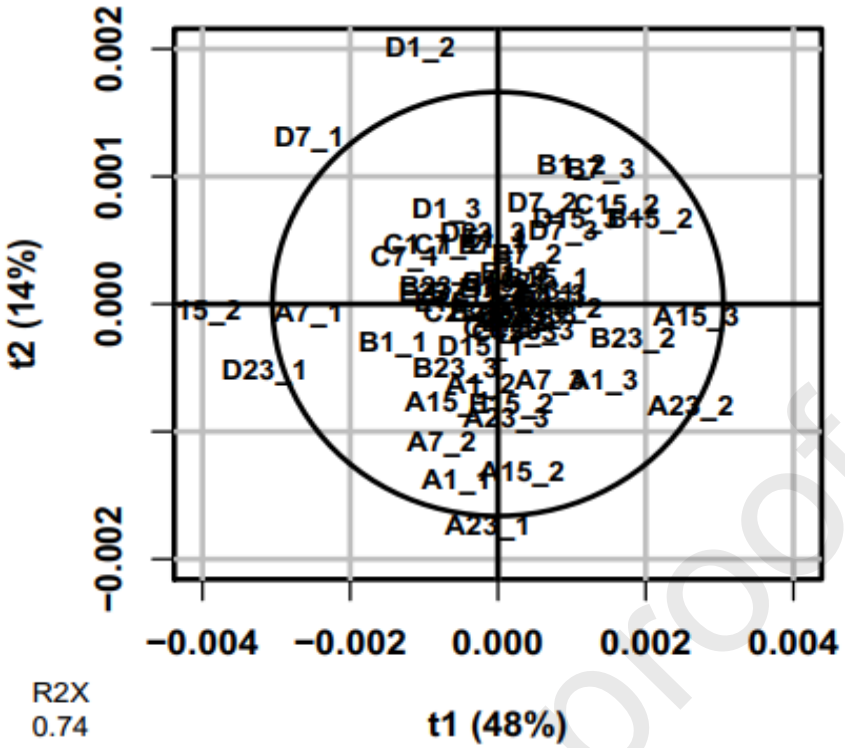

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765 **Figure 1 –** PCA scores plot of yoghurt produced under different conditions of pressure (0.1, 10, 20, 30 and
766 40 MPa) obtained by 1D ^{1}H NMR. *Legend of sample name code:* Letters represent the pressure of
767 fermentation A to E means 0.1 to 40 MPa, the first number at the right of letter mean the day of storage (1,
768 7, 15 or 23) and the second number represent the number of replica (1, 2 or 3).

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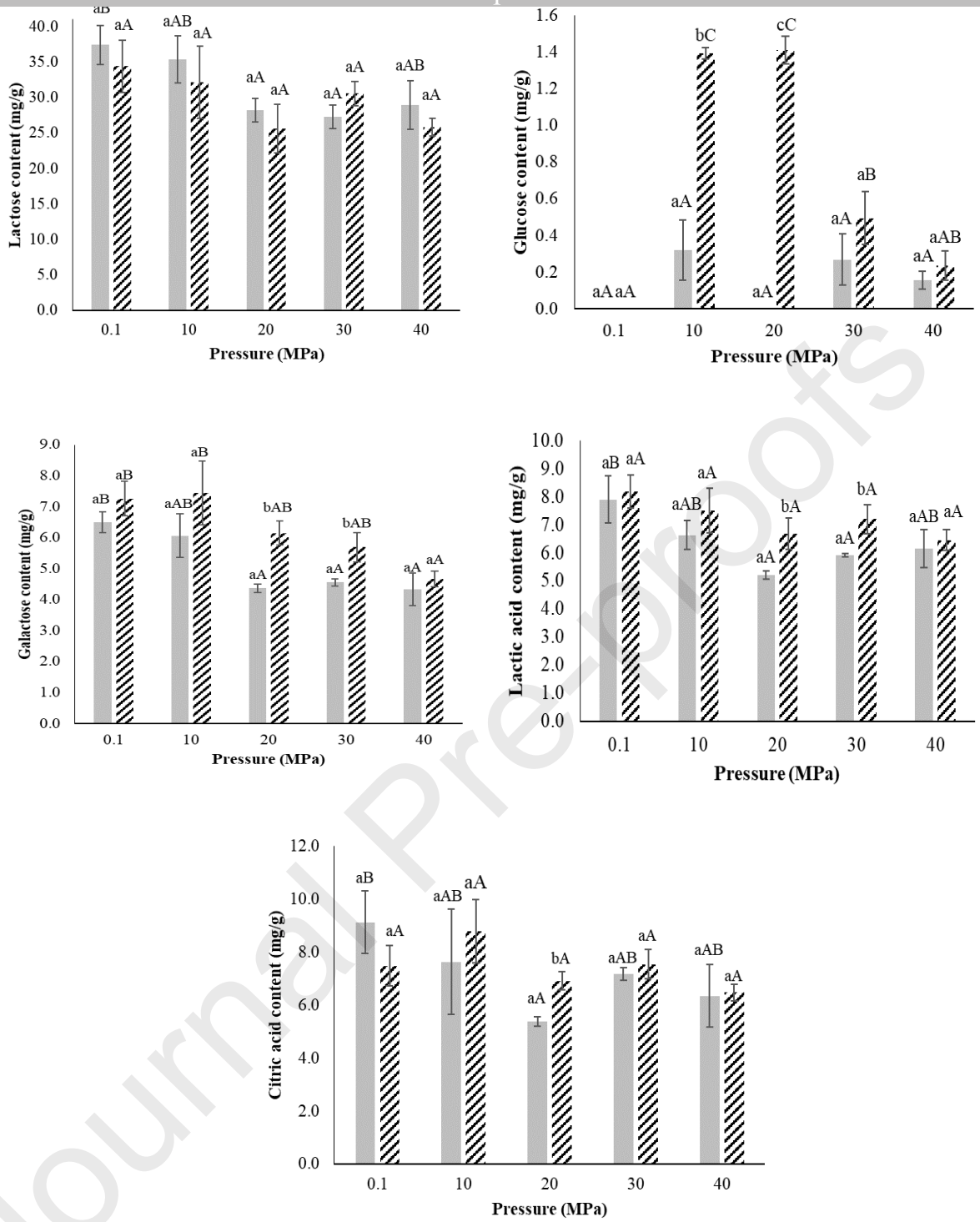

Figure 2 – Lactose (A), glucose (B), galactose (C), lactic acid (D) and citric acid (E) content of each yoghurt fermented under pressure (0.1, 10, 20, 30 and 40 MPa) for the 1st ( ) and the 23rd ( ) day of storage. Different lower (a-b) and upper (A-B) case letters indicate statistical differences ($p < 0.05$) between storage periods and pressures, respectively.

Tables:

Table 1 - List of the principal metabolites identified in samples by comparison with databases and an appropriate software (Chenomx), with the respective chemical shifts.

Compounds	Chemical shift (ppm)	Compounds	Chemical shift (ppm)
2,3-butanediol	1.12 – 1.16	Sugars_1	3.10 – 4.10
Acetate	1.87 – 1.95	Sugars_2	4.42 – 4.48
Acetaldehyde	2.03 – 2.08	Sugars_3	4.56 – 4.60
Acetoin	2.21 – 2.24	Sugars_4	4.62 – 4.70
Citrate	2.60 – 2.85	Sugars_5	4.76 – 43.82
Diacetyl	2.37 – 2.38	Sugars_6	5.21 – 5.245
Formate	8.41 – 8.43	Sugars_7	5.25 – 5.29
Lactate	1.24 – 1.28; 4.14 – 4.22	Sugars_8	5.36 – 5.455
Pyruvate	2.55 – 2.60	Sugars_9	6.185 – 6.20
Alanine	1.46 – 1.49	Unknown_1	0.75 – 1.00
		Unknown_2	3.02 – 3.05

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833 **Table 2 –** Changes in fatty acid (FA) group profile along storage, expressed in percentage (%) of each
834 yoghurt fermented under different pressures (0.1, 10, 20, 30 and 40 MPa) (n=3).

	Pressure (MPa)	0.1		10		20		30		40	
	Day of storage	1st	3rd	1st	3rd	1st	3rd	1st	3rd	1st	3rd
Fatty acids (%)	SCFA +SFA	5.6	6.0	6.1	6.6	6.3	6.8	7.2	7.0	7.6	7.2
	MUFA	0.9	0.4	9.9	9.3	9.6	9.2	8.7	8.8	8.8	9.2
	PUFA	.5	.5	.1	.2	.1	.0	.0	.1	.6	.7
	Total FA n-3	.1	.2	.6	.7	.7	.6	.6	.7	.2	.2

Total FA n-6	(.7	(.6	(.7	(.6	(.6	(.4	(.3	(.4	(.3	(.3
FA cis	0.6	0.5	1.2	1.0	1.1	0.8	0.4	0.6	9.6	9.7
FA trans	2.2	1.9	1.2	0.8	1.0	0.8	0.8	0.8	1.2	1.4

Note: Short-chain fatty acids and short fatty acids (SCFA+ SFA) monounsaturated fatty acids (MUFA); polyunsaturated fatty acids (PUFA); Total fatty acids omega 3 (Total FA *n*-3); Total fatty acids omega 6 (Total FA *n*-6); Total cis unsaturated fatty acid (FA *cis*); Total trans unsaturated fatty acid (FA trans);

Table 3 - Lipid quality indices of yoghurt fermented under 0.1, 10, 20, 30 and 40 MPa for the 1st and 23rd day of storage (n=3).

Quality parameter	**IA**		**IT**		***n*-6/*n*-3**		**MUFA/ PUFA**		**PUFA / (SCFA+SFA)**	
Pressure (MPa)	**1st**	**23rd**	**1st**	**23rd**	**1st**	**23rd**	**1st**	**23rd**	**1st**	**23rd**
0.1	0.06	0.06	0.03	0.03	0.32	0.29	3.04	2.71	0.23	0.22
10	0.06	0.06	0.03	0.03	0.28	0.26	9.69	9.08	0.25	0.25
20	0.06	0.06	0.03	0.03	0.25	0.24	9.51	9.70	0.25	0.24
30	0.06	0.06	0.03	0.03	0.25	0.26	9.47	9.13	0.23	0.24
40	0.06	0.06	0.03	0.03	0.29	0.28	1.86	1.66	0.21	0.21

Index of atherogenicity (IA); index of thrombogenicity (IT); Omega-6/omega-3 (*n*-6/*n*-3); monounsaturated/polyunsaturated fatty acid (MUFA/PUFA); polyunsaturated/short-chain fatty acids and saturated fatty acid (PUFA/SFA);

Highlights

- Yoghurts fermented under pressure presented distinct features.
- 2,3-butanediol, acetoin, diacetyl and formate content is influenced by pressure.
- Mean proportions of lactose/lactic acid were similar along storage.
- Yoghurts fermented at 40 MPa had the less content in sugars and fatty acids.