Universidade de Aveiro Departamento de Química

Ano 2013

MARIA JOÃO PINHO MOTA

EFEITO DA ALTA PRESSÃO NA PRODUÇÃO DE IOGURTE PROBIÓTICO

EFFECT OF HIGH PRESSURE ON PROBIOTIC YOGURT PRODUCTION

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, realizada sob a orientação científica do Doutor Jorge Manuel Alexandre Saraiva, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro e da Doutora Ivonne Delgadillo Giraldo, Professora Associada com Agregação do Departamento de Química da Universidade de Aveiro.

Dedico este trabalho aos meus pais, como forma de gratidão pelo incansável apoio demonstrado, não só durante esta etapa, mas ao longo de toda a minha vida.

o júri

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palavras-chave Alta pressão, iogurte, fermentação, bactérias ácido-láticas, probióticos.

Resumo

O principal objetivo deste trabalho incide na análise do efeito da alta pressão nas bactérias ácido láticas e probióticas, durante o processo fermentativo de produção de iogurte. Nesse sentido, diversas combinações de pressão/tempo foram testadas e vários parâmetros físico-químicos e microbiológicos foram avaliados. De modo a monitorizar a fermentação lática, mediu-se o pH, a acidez titulável e a concentração de açúcares redutores. Para além disso, efetuou-se ainda a quantificação de D-glucose, L- e D-ácido lático, acetaldeído e etanol na amostra. Por fim, os microrganismos relevantes neste produto (*Streptococcus thermophilus*, *Lactobacillus bulgaricus* e *Bifidobacterium lactis*) foram também quantificados.

Numa fase inicial, concluiu-se que os parâmetros de monitorização da fermentação eram claramente afetados pela pressão. Com o aumento da pressão, verificou-se uma menor variação do pH, da acidez titulável e da concentração de açúcares redutores ao longo do tempo, mostrando que a alta pressão reduz a velocidade da fermentação lática, aumentando o tempo de fermentação. A 5 MPa foi ainda possível atingir o pH requerido para que o produto seja classificado como iogurte; por outro lado, a 100 MPa o processo fermentativo não ocorreu. Adicionalmente, algumas amostras foram submetidas a um pré-tratamento sob alta pressão, previamente à fermentação a 0.1 MPa. Os resultados mostram que as bactérias *starter* não sofreram danos irreversíveis durante o pré-tratamento, uma vez que, posteriormente, estas recuperaram a sua atividade fermentativa. Observou-se que, nas amostras expostas a 100 MPa, a concentração de D-glucose aumentou ao longo do tempo, possivelmente indicando que nestas condições as células expelem D-glucose. Relativamente aos isómeros de ácido lático, verificou-se que, para todas as condições testadas, as amostras apresentavam uma concentração de L-ácido lático superior à do isómero D-. De um modo geral, a concentração de acetaldeído mostrou tendência para aumentar ao longo do tempo de fermentação, atingindo valores de concentração superiores no caso das amostras a 5 MPa. Verificou-se ainda que, quer à pressão atmosférica quer sobre alta pressão, não ocorreu produção significativa de etanol durante a fermentação do iogurte. No que diz respeito à análise microbiológica, concluiu-se que a pressão inibiu o crescimento (e causou destruição celular, em alguns casos) dos três microrganismos estudados. Verificou-se que a *S. thermophilus* corresponde à bactéria com maior resistência à pressão, enquanto a *L. bulgaricus* é a mais sensível. A *B. lactis* mostrou capacidade para crescer a 5 MPa, mas sofreu 2 reduções logarítmicas quando exposta a 100 MPa.

Keywords High pressure, yogurt, fermentation, lactic acid bacteria, probiotics.

Abstract

The main goal of this work corresponds to the analysis of the effect of high pressure in lactic acid and probiotic bacteria, during yogurt production. To that purpose, different combinations of pressure/time were tested and several physicochemical and microbiological parameters were evaluated. To monitor lactic acid fermentation, pH, titratable acidity and concentration of reducing sugars were measured. In addition, the quantification of D-glucose, L- and D-lactic acid, acetaldehyde and ethanol was also performed, to better understand the implications of high pressure in some biochemical and nutritional yogurt properties. At last, it was also important to evaluate some microbiological parameters, in this case the microbial counts of *Streptococcus thermophilus*, *Lactobacillus bulgaricus* and *Bifidobacterium lactis.*

Firstly, it was observed that the fermentation monitoring parameters were clearly affected by pressure. With the increasing pressure it was observed a lower variation in pH, titratable acidity and reducing sugars concentration over time, indicating that pressure reduces the lactic acid fermentation rate (and increases the fermentation time). At 5 MPa it was still possible to achieve the pH required to obtain yogurt, while at 100 MPa the fermentation process was ceased.

Additionally, some samples were subjected to a high pressure pre-treatment (previously to fermentation at atmospheric pressure) and the results showed that the fermentative cells have not suffered severe damage during the pre-treatment, since after that, at atmospheric pressure, its metabolic activity was recovered. It was observed that D-glucose concentration increased over time in samples subjected to 100 MPa, showing that cells are expelling D-glucose (formed by lactose hydrolysis, intracellularly) to the extracellular medium. Relatively to lactic acid isomers it was concluded that, to all different tested pressure conditions, the yogurt samples had a higher concentration of L-lactic acid relatively to the D-isomer. Acetaldehyde content tended to increase over the fermentation time in all evaluated samples, but with a higher rate at 5 MPa. Furthermore, it was verified that there was no ethanol production during yogurt fermentation, neither at atmospheric pressure, nor under high pressure. In the case of microbiological analysis, it was concluded that high pressure inhibited the growth (and caused destruction, in some cases) of all three evaluated microorganisms. It was observed that *S. thermophilus* corresponds to the most pressure resistant, while in contrast *L. bulgaricus* is the most pressure sensitive bacteria. *B. lactis* was capable to grow under 5 MPa, but it was observed a 2 logarithmic reduction at 100 MPa.

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I. INTRODUCTION

1. Dairy Products and Yogurt

Fermentation is one of the oldest methods used for the transformation of milk into products with an extended shelf life [1]. However, the fundamental reasons for the great development and acceptance of fermented foods in the last decades can be attributed not only to preservation, but also to improved nutritional properties, better flavor/aroma, upgrading of substrates to higher value products and improved health aspects [2-4]. All these properties are the result of the activity of a population of bacterial strains, mainly lactic acid bacteria (LAB), which use the lactose in milk to produce lactic acid and other important compounds in fermented dairy products [4]. Depending on the microorganisms involved, fermentation may proceed via the glycolysis pathway with the almost exclusive formation of lactic acid (homofermentation), via the pentose phosphate pathway with formation of lactic acid, acetic acid or ethanol, and possibly CO_2 (heterofermentation) or via both pathways [5].

Lactic acid fermentation is involved in the manufacturing of a wide range of dairy products with a diversity of flavor and textural attributes, including cheese, yogurt, buttermilk, butter, acidophilus milk, sour cream, and others [4]. Yogurt is defined as a coagulated milk product that results from the fermentation of lactic acid in milk by symbiotic cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*, which must be found alive in the final product (≈10 million CFU/g of yogurt) [6, 7]. These two organisms live together symbiotically, each producing compounds that promote the growth of the other [5, 8]. During fermentation, the production of lactic acid by LAB decreases the pH of the milk, causing coagulation of the caseins. As the pH decreases to less than 5.3, colloidal calcium phosphate is solubilized from the casein micelle, causing the micelles to dissociate [4]. Then at caseins isoelectric point, pH 4.6, the destabilized casein micelles aggregate into a three-dimensional network structure. The resulting gel, which is somewhat fragile in nature, provides the yogurt characteristic structure [4, 9, 10].

Nowadays several types of fermented milks are available, with new products emerging in the market every day [7, 11, 12]. The contribution of biotechnology has been very important during the last few years as it offers the possibility of selecting and

using new sources, to increase the yield of sources already used, to introduce specific functional properties in raw materials or ingredients, to improve the nutritional value and the bioavailability of nutrients and flavor [3].

1.1.Yogurt Production

Yogurt manufacturing methods, raw materials and formulations vary widely from country to country, resulting in products with a diversity of flavor and texture characteristics [4].

Figure 1 outlines the steps involved in the processing of yogurt, which is extensively described in literature. Firstly, milk is standardized to the desired fat and milk solids-not-fat (MSNF) content. The addition of non-fat milk powder increases the protein content, improving the body and decreasing the syneresis of the final product. Syneresis corresponds to the expelling of interstitial liquid due to association of the protein molecules and shrinkage of a gel network and it is undesirable in yogurt [4].

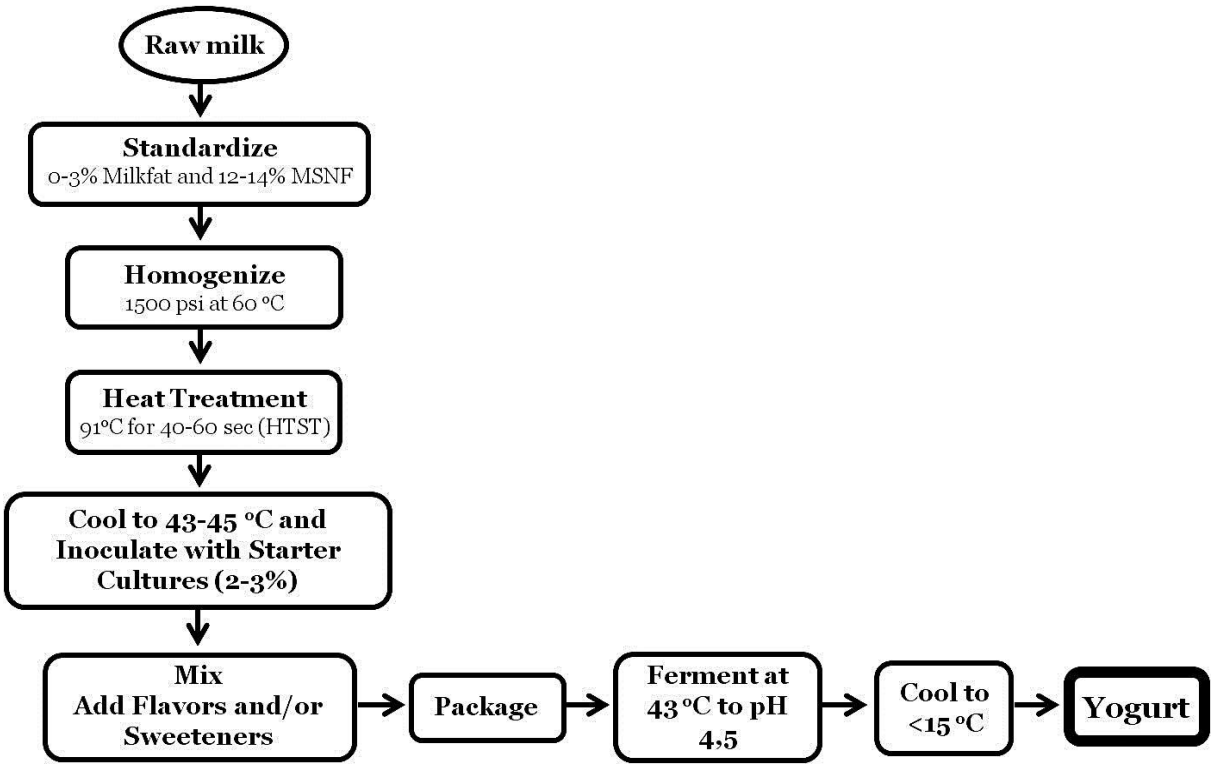

Figure 1. Processing scheme for yogurt production (Adapted from [4]).

During milk homogenization the size of the fat globule decreases, leading to the stabilization of the milk fat in the water phase. The homogenized milk is then submitted to a heat treatment (pasteurization) which eliminates pathogenic microorganisms and

reduces the oxygen in the milk, providing a good growth medium for the starter cultures. Enzymes and the major whey proteins, including β lactoglobulin and α lactalbumin, but not the casein proteins, are also denatured by the heat treatment [1, 4].

The treated milk is then cooled for inoculation of the starter cultures (1.5–3%) and the incubation is conducted at 42–45^{o}C for about 3 hours (180 minutes) [4, 5]. When the goal is to produce probiotic yogurt, the strains with the desired properties are added to the starter culture and they are both inoculated to milk at the same time. It is generally considered that the yogurt is ready when it reaches a pH near to 4.5 or a titratable acidity of 0.7–1.1% of lactic acid [4, 5]. When these values are obtained, it is important to cool the yogurt to stop fermentation and to maintain its structure [13].

Besides the traditional set yogurt (which production was described in this section), several products with a great variety of characteristics emerged in the market and enjoy a high popularity among the consumer, such as in the case of stirred and liquid yogurt, or even fruit yogurts (with addition of fruits and fruit pastes).

1.2.Starter Bacteria

As previously said, yogurt contains a thermophilic starter culture comprised by *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus. S. thermophilus* was originally described by Orla-Jensen (1919) [14] and stands apart from the other streptococci and especially lactic streptococci (designated as lactococci). It is exclusively isolated from the dairy environment and it ferments lactose, sucrose, glucose and sometimes galactose. This microrganism is characterized by its thermoresistance, since it shows a rather high growth temperature, which may reach 50-52^{o}C [15]. The other microbial yogurt starter, *L. bulgaricus*, was also firstly described by Orla-Jensen (1919) [14]. It ferments a few carbohydrates, such as glucose, lactose, fructose, and sometimes galactose or mannose. Just as happens with *S. thermophilus*, *L. bulgaricus* has a high growth temperature, up to 48 or 50^{o}C [16]. *L. bulgaricus* are rod with rounded ends shape, while *S. thermophilus* has a spherical to ovoid shape with irregular segments [15]. Both are Gram-positive, facultative anaerobic, non-motile and non-spore-forming bacteria [16]. Figure 2 shows the Gram stain and the shape of *L. bulgaricus* and *S. thermophilus* isolates.

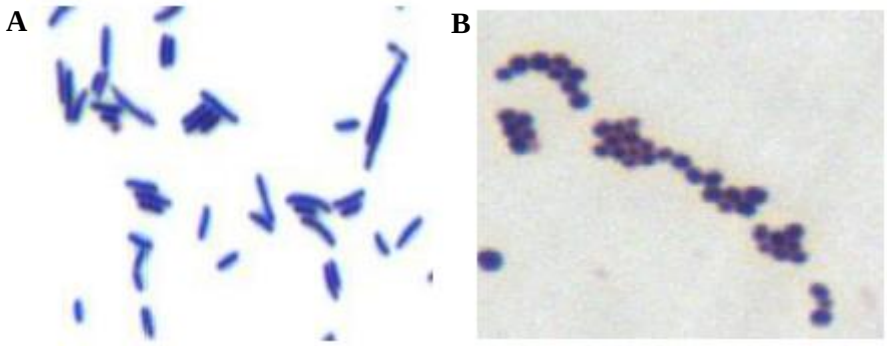

Figure 2. Microscopic images of *Lactobacillus bulgaricus* (A) and *Streptococcus thermophilus* (B) [17].

1.2.1. Influence of Oxygen on LAB Growth

As mentioned above, LAB are facultative anaerobes, then with a preference for anaerobic conditions [18]. They cannot synthesize porphyrins and consequently they do not synthesize cytochromes or catalase. Oxygen is often used for the formation of hydrogen peroxide (H_2O_2), a compound that is highly toxic for bacteria which do not contain catalase to break it down, as in the case of the most LAB [19]. While *S. thermophilus* tolerates controlled amounts of oxygen, *L. bulgaricus* is among the least oxygen tolerant LAB, since it produces a very large amount of H_2O_2 which inhibits its own growth as well as the growth of some other bacteria [19, 20].

1.2.2. Metabolic Pathways of Lactose, Glucose and Galactose Utilization

Some of the main metabolic pathways of lactic acid bacteria are schematized in Figure 3. For transport of lactose into the cell, LAB usually possess two different systems: a phosphotranspherase system (PTS) and a permease system, both of which require energy [21]. Most thermophilic starter bacteria, such as streptococci and lactobacilli, use the permease system for lactose transport. In this system, the energy is derived from adenosine triphosphate (ATP) and lactose is transported without any previous transformation [22, 23]. Once lactose is transferred inside the cell, the enzyme involved in its initial metabolism is β galactosidase (β-gal). The β-gal hydrolyses lactose to glucose and galactose, which are subsequently fermented via the Embden–Meyerhof–Parnas (glycolytic) and Leloir pathways, respectively. In the Leloir pathway, galactose is transformed to glucose-1-P, and this product is further metabolized through the glycolytic pathway, with lactic acid as the end-product [24]. Most of the *S. thermophilus* strains are not able to use galactose (Gal^- phenotype) and release the galactose moiety to the extracellular medium [25]. However, some Gal^+ strains have

been isolated [26, 27]. Concerning *L. bulgaricus*, only the glucose moiety of lactose is generally metabolized and galactose is released into the growth medium. However, some strains can use galactose in a growth medium containing limiting concentrations of lactose [7, 28].

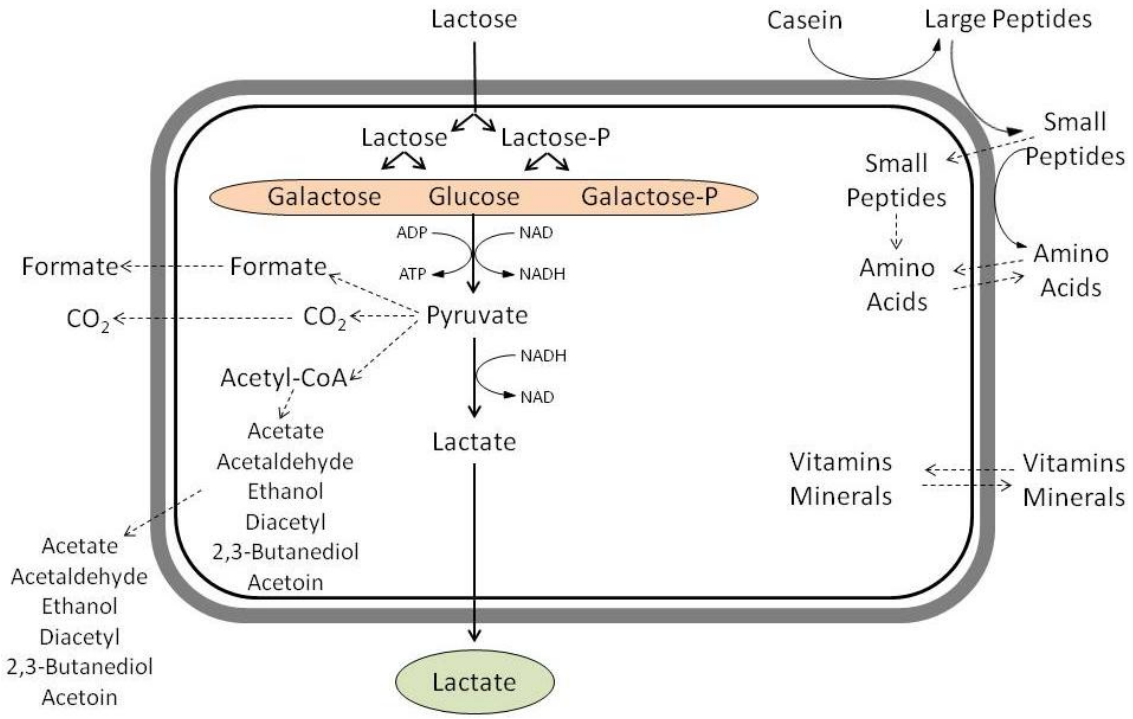

Figure 3. Representation of the main metabolic pathways of LAB (Adapted from [29]).

During yogurt fermentation *S. thermophilus* produces mainly L-lactic acid, while *L. bulgaricus* produces mainly D-lactic acid and, in consequence, both lactic acid isomers are simultaneously produced in yogurt. Since D-lactic acid is metabolized very slowly in man (and may cause metabolic disorders if ingested in excess), the industrial starters used in yogurt production must have a low proportion of *L. bulgaricus* [5, 7].

1.2.3. Sucrose Utilization

As previously referred, *L. bulgaricus* is not capable to degrade and consume sucrose, contrarily to what is verified to *S. thermophilus*. During the growth of *S. thermophilus* on sucrose, both glucose and fructose moieties are used. However, fructose accumulates in the growth medium even when the strain can use it [30]. An inhibitory effect of high sucrose content in milk (10-12%) on the growth of yogurt

bacteria has often been reported, due to both an adverse osmotic effect of the solutes in milk and a low water activity [1].

1.2.4. Proteolytic Activity

In yogurt, proteolysis is not relevant for organoleptic properties. On the other hand, proteolytic activity is greatly involved in both nutrition and interactions of yogurt bacteria [7]. During proteolysis several peptides with different biological activities can be formed, for instance, opiate activity and hypotensive, immune-stimulating or antimicrobial effects [5].

In addition, it is known that the low molecular weight peptide fraction of milk is an important nitrogen source for yogurt bacteria, since LAB cannot synthesize essential amino acids. Therefore, they require an exogenous nitrogen source and utilize peptides and proteins in their growth medium by more or less complete enzyme systems [7].

1.2.5. Interactions between Yogurt Bacteria

As starter cultures for yogurt production, LAB species display symbiotic relations during their growth in milk medium [31]. Thus, a carefully selected mixture of LAB species is used to complement each other and to achieve a remarkable efficiency in acid production [32]. A positive interaction is generally observed between *S. thermophilus* and *L. bulgaricus* in mixed culture, leading to the stimulation of growth and acid production of both bacteria compared to their single-strain cultures [33-35]. Mixed yogurt cultures may also stimulate the production of some metabolites such as acetaldehyde and influence carbohydrate utilization [36, 37]. For instance, one *L. bulgaricus* strain which cannot use galactose in pure culture metabolizes this sugar when it is associated with one strain of *S. thermophilus* [38, 39]. Another example of symbiotic behavior is related to the proteolytic activity of the starter strains: *S. thermophilus* does not possess substantial extracellular proteolytic activity and the amino acid and free peptide content of milk is not high enough to promote its full growth. *L. bulgaricus* proteases break down caseins and supply the *Streptococcus* with amino acids and peptides [34, 40, 41]. In conclusion, the interaction between yogurt bacteria is a good example of integrated metabolism in a mixed culture of LAB [7].

1.3.Biochemical and Nutritional Composition of Yogurt

The nutrient composition of yogurt is based on the nutrient composition of the milk from which it is derived. Other variables that play a role during processing of milk, including temperature, duration of heat exposure, exposure to light, and storage conditions, also affect the nutritional value of the final product. In addition, the changes in milk constituents that occur during lactic acid fermentation influence the nutritional and physiologic value of the finished yogurt product [32].

Dairy products are an exclusive natural source of lactose in human diet. Before fermentation, the lactose content of the yogurt mix is generally ≈ 6% [31, 32]. However, during fermentation starter and probiotic bacteria hydrolyze between 20 and 30% of lactose to its absorbable monosaccharide components (glucose and galactose), through the activity of β galactosidase [6, 32]. Then, a portion of the glucose moiety is converted to lactic acid (in the most cases, galactose is expelled from the cell). The lower lactose concentration in yogurt than in milk partially explains why yogurt is better tolerated than milk by persons with lactose maldigestion [32, 42-44].

Casein constitutes about 80% of the total protein content and its coagulation comprises the central process in conversion of milk to yogurt and may also contribute to the greater protein digestibility of yogurt compared to milk [10, 32]. In addition to casein, milk contains other proteins which remain soluble at pH values low enough to cause agglomeration of casein. These are known as whey proteins and contribute about 20% of the total protein content. The principal whey proteins are β lactoglobulin, α lactoalbumin, blood serum albumin (BSA) and immunoglobulin, which constitute, respectively, about 50, 20, 10 and 10% of the total whey proteins in bovine milk [10, 45]. In addition to being a good source of protein, yogurt is also an excellent source of calcium and phosphorus. In fact, dairy products such as milk, yogurt and cheese provide most of the highly bioavailable calcium in the typical Western diet [32, 46].

Concerning to the lipid fraction, it is known that the free fatty acid content of yogurt differs only slightly from that of milk [7, 47]. Since lipolytic activity is generally low, minor amounts of free fatty acids are released during lactic acid fermentation and are not significant in terms of flavor [31]. However, yogurt has been shown to have a higher concentration of conjugated linoleic acid (CLA), a long-chain hydrogenated derivative of linoleic acid, than does the milk from which the yogurt was obtained [48]. It was then hypothesized that hydrogenation occurs during fermentation of milk and results in higher concentrations of CLA in the final product [49].

1.3.1. Flavor Compounds

Starter cultures are primarily responsible for the production of the flavor compounds which contribute to the aroma of yogurt. These compounds may be divided into four main categories [1]:

- Non-volatile acids (e.g. lactic, pyruvic, oxalic or succinic);
- Volatile acids (e.g. formic, acetic, propionic or butyric);
- Carbonyl compounds (e.g. acetaldehyde, acetone, acetoin or diacetyl);
- Miscellaneous compounds (certain amino acids and/or constituents formed by thermal degradation of protein, fat or lactose).

The typical flavor of yogurt is mostly due to carbonyl compounds, e.g. acetaldehyde, acetone and diacetyl, produced by *S. thermophilus* and *L. bulgaricus.* Acetaldehyde is considered as the major flavor component of yogurt [50-52], while high concentrations of this compound in other dairy products (cheese or cream) lead to flavor defects described as "green" or "yogurt-like" [53]. Diacetyl contributes to the delicate, full flavor of yogurt and seems to be important when the acetaldehyde content is low [54]. Other carbonyl compounds, such as 1-octen-3-one and 1-nonen-3-one, have also been detected as an important odorant in yogurt [5].

In addition to carbonyl substances, many volatile compounds have also been identified in yogurt, such as volatile fatty acids [51, 55] and several compounds derived from the thermal degradation of lipids, lactose and proteins, such as aldehydes, ketones, alcohols, lactones, sulfur compounds [56].

2. Probiotic Yogurt

Nowadays, consumers are aware of the link among lifestyle, diet and good health, which explains the emerging demand for products that are able to enhance health beyond providing basic nutrition. The list of health benefits accredited to functional food continues to increase and probiotics are one of the fastest growing categories within food for which scientific research have demonstrated therapeutic evidence [57].

Although the concept of probiotics was introduced in the early 20^{th} century, the term was not coined until the 1960s and its definition has evolved through the years. Many definitions of the term probiotic have been published, however the most widely

accepted is that "probiotics are live microorganisms, administrated in certain quantities that confer health benefits to the host" [58].

During the last two decades, probiotics have been added in different food matrices, but especially in fermented milks [59, 60]. Probiotic yogurt occupies a very strong position in the dairy products market, and there is a clear trend to increase its consumption in the next few years [61]. The global market of probiotic ingredients, supplements and food was worth $14.9 billion in 2007 and it is expected to reach 19.6 billion in 2013, representing a compound annual growth rate of 4.3 % [62]. According to some authors [63, 64] the most popular probiotic strains are represented by the genera *Lactobacillus, Streptococcus* and *Bifidobacterium*. However some strains of other microbial genera, such as enterococci and yeast (e.g. *Saccharomyces boulardii*) are also thought to have probiotic properties.

2.1.*Bifidobacterium* spp.

Bifidobacteria were first isolated and described in 1899–1900 by Tissier, who described rod-shaped, non-gas-producing, anaerobic microorganisms, present in the faeces of breast-fed infants, which he termed *Bacillus bifidus* [57]. Bifidobacteria are generally characterized as Gram-positive, non-spore-forming, non-motile and catalase-negative anaerobes [65]. They have various shapes, including short, curved rods, club-shaped rods and bifurcated Y-shaped rods.

Presently, 30 species are included in the genus *Bifidobacterium*, 10 of which are from human sources (dental caries, faeces and vagina), 17 from animal intestinal tracts or rumen, two from wastewater and one from fermented milk [66]. From all these, only six species of bifidobacteria have attracted attention in the dairy industry: *B. adolescentis, B. breve*, *B. bifidum, B. infantis*, *B. animalis* subsp. *lactis (*so-called *B. lactis)* and *B. longum*. It is important to note that in all these cases the organisms have been isolated from human subjects, and this restriction is based on the assumption that, if an isolate is of human origin, then it should become implanted and metabolize in the colon of another human. The validity of this idea remains open to debate [1].

Different *Bifidobacterium* species utilize different types of carbohydrates, but one key enzyme is always involved, which is fructose-6-phosphate phosphoketolase (F6PPK), also known as "bifidus shunt", and it can be used to identify the genus. The fermentation of two molecules of glucose leads to two molecules of lactic acid and three molecules of acetate [1]. Although bifidobacteria strains ferment lactose, they are

unable to generate enough lactic acid for the manufacture of fermented foods with characteristic aroma and flavor, especially when used as a monoculture [67]. Another limitation is the fact that bifidobacteria are not well adapted to fermented milk and suffer in the presence of oxygen. Therefore, an important selection criteria for specific strains is the growth and survival in acidified and partly aerobic conditions [57].

Bifidobacteria are microorganisms of paramount importance in the active and complex ecosystem of the intestinal tract of humans and other warm-blooded animals [65]. Although they are relatively minor components of the normal gastrointestinal microbiota in human adults, research indicates that some strains can promote or provide several health-related functions, including host resistance to infectious microbes, anti-carcinogenic activities, and improved nutritional efficiency [68, 69].

2.2.Therapeutic Properties of Probiotic Strains

In order to ensure the health promoting effect of probiotic yogurt on the human body, it is necessary to maintain the proper amount of live probiotic bacteria (not less than 10 million CFU/g of the product) throughout the product's shelf life [70]. Several health benefits are attributed to the ingestion of probiotic-containing foods, some of them have been proven scientifically (Figure 4) and others still require further studies in humans [71].

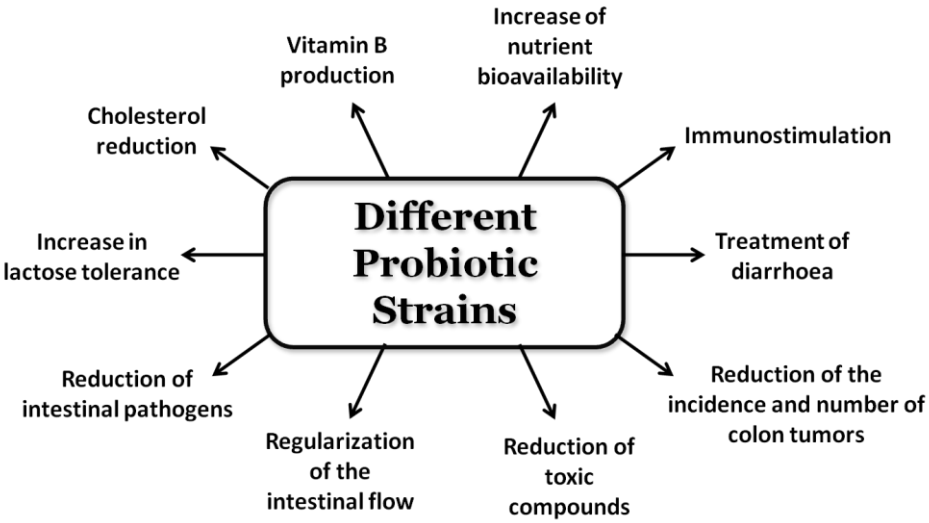

Figure 4. Some documented physiological benefits of functional foods containing probiotic bacteria (Adapted from [71]).

Some of the most relevant physiological effects attributed to probiotic consumption are described below, together with a few published studies reporting this beneficial effect.

2.2.1. Gut Defense

One of the main physiological benefits of probiotics is attributed to a non-immunologic gut defense against specific groups of microorganisms, through the stabilization of the gut microflora [3, 72]. However probiotic bacteria are shown to promote the endogenous host defense mechanisms as well, by enhancing humoral immune responses and thereby promoting the intestine's immunologic barrier [73].

Some bifidobacteria strains have been shown to adhere and to colonize in different types of cultured intestinal epithelial cells [74]. Some authors reported that some of them are able to stabilize the intestinal microbiota during and after antibiotic therapy, and to modulate the immune system, protecting against chemically induced intestinal inflammation and reducing symptoms of colitis [75, 76].

In a randomized double-blind placebo-controlled study, the oral application of *B. lactis Bb12* to preterm infants, who are prone to intestinal infections and necrotizing enterocolitis, improved several health-associated markers. In the probiotic group, the fecal pH was significantly lower than in the placebo group, in accordance with the higher fecal concentrations of acetate and lactate in the infants receiving *B. lactis Bb12*. Fecal calprotectin was lower in the probiotic group, suggesting a reduced inflammation of the intestinal mucosa. A higher fecal IgA level in the *B. lactis Bb12* group indicates an improved mucosal antibody-based defense [77].

2.2.2. Cholesterol Reduction

Probiotic strains have a major role to play in the cholesterol lowering mechanism. The mechanisms can be direct (by decreasing the intestinal absorption of dietary cholesterol) or indirect (by deconjugating the cholesterol to bile acids, thereby enhancing the fecal excretion of steroids and reducing serum cholesterol) [3, 78].

Gilliland *et al.* (1985) [79] conducted an experiment on pigs which were fed with high cholesterol diet followed by feeding with probiotic strains of *L. acidophilus* P-47 and RP-32. From the fifth day onwards, the authors verified a significant lower serum cholesterol concentration in pigs fed with *L. acidophilus* P-47, relatively to the control group. The potential hypocholesterolemic effect of probiotics sparked much

interest based on evidence from animal work and human studies, when amounts of 0.5–5 L of yogurt per day were consumed. However, the ingestion of realistic quantities of yogurt or probiotics in man has not been shown to reduce cholesterol levels significantly [80, 81].

2.2.3. Anticancer Effects

Diets high in animal protein and fat appear to increase the susceptibility to colon cancer, apparently through conversion of procarcinogens to carcinogens, by the intestinal microflora [78]. Studies on the effect of probiotic consumption on cancer appear to be promising, since animal and *in vitro* studies indicate that probiotic bacteria may reduce colon cancer risk [82]. There is some evidence that probiotics can interfere at various stages of the cancer process, such as prevention of DNA damage in the colon [83], suppression of pre-neoplastic changes in the colon [84] and suppression of colon tumours in animals [85].

Goldin *et al*. (1977) [86] demonstrated that *L. acidophilus* supplementation in man changed the intestinal bacteria activity, leading to a reduction of beta-glucuronidase, nitro-reductase and azo-reductase activity and bacterial enzymes that are associated with the conversion of procarcinogens to proximal carcinogens. Fermented milk with viable yogurt strains of *L. helveticus* or *Bifidobacterium* sp. have shown an effect on colon cancer cell growth and differentiation with co-culture *in vitro* [87]. *L. casei* has been shown to decrease the activity of enzymes related to the risk of colon cancer and to inhibit mutagenicity [88]. Additional studies are needed to further investigate the effect of probiotic and LAB in reducing the risk of cancer [89].

2.3.Challenges

There are two major areas of functionality that are required for a probiotic. On one hand, there is the documented health benefit and, on the other, the technological properties of the strain [90]. The employment of probiotic bacteria in dairy industries constitutes a challenge due to some characteristics of food matrix that requires enhancement for probiotic viability and stability [91]. Given the limited proteolytic activity of probiotic bacteria on milk casein, it is often necessary to supplement the dairy matrix with sources of nitrogen such as hydrolyzed protein, whey derivatives, and amino acids for use by the probiotic bacteria. This strategy has been extensively

performed [92-95] and positive impacts have been observed on the viability of probiotic strains.

The low pH values that probiotic bacteria are exposed to, during the processing of dairy products, is also a matter of concern. The simplest technological solution is to promote a previous strain exposure to lower pH for a short period of time, thereby inducing a tolerance of the microorganism [96]. This strategy has been successfully applied [97] and it was concluded that it may also favor probiotic metabolic function and survival in the gut [3, 96].

In general, it is prudent that the probiotic strains are compatible with the starter cultures conventionally used in dairy products, avoiding problems such as inhibition by acid, peroxide, bacteriocins or other metabolites. Inhibition problems between starter and probiotic cultures have been reported and cannot be neglected [98, 99]. There seems to be a great compatibility between certain strains that allows the proper development of both in the product matrix [100]. It also has been noted that the proper compatibility may influence adherence to the intestinal mucosa, which may directly influences the product functionality [101].

A probiotic in a dairy product must be balanced between the minimum number of cells to confer health effects but also taking into account the sensory acceptance by consumers. Bifidobacteria produce acetic and lactic acids in the proportion of 3:2 and the taste and aroma of acetic acid provide extremely undesirable off-flavors to dairy products, requiring the use of flavoring agents. An effective alternative to overcome this possible undesired consequence is the addition of microencapsulated cells of probiotic cultures to dairy food products [71, 102].

Safety is another important criterion in the selection of a probiotic strain. In order for probiotics to be considered as safe, the strains should be considered to be non-pathogenic, non-toxic/carcinogenic and not absorbed in digestive tract/not invasive. In addition they should be genetically stable and do not carry any transmissible antibiotic resistance genes [3, 90].

3. High Pressure Technology

Hydrostatic pressure is a key physical parameter in the biosphere that ranges from 0.1 MPa (atmospheric pressure), at sea level, to more than 110 MPa, in ocean depths. Pressure is considered a variable of life which has influenced the evolution and

distribution of both microorganisms and macroorganisms. Pressures of different magnitudes exert different effects on organisms and the ability to adapt to pressure changes of one kind or another is a characteristic of all life [103-105].

High pressure (HP) is an emerging technology, which is receiving a great deal of attention in the last years, and exerts its effects on biological systems in accordance with the following operating principles [106]:

- **Le Chatelier's principle**: Any chemical reaction which is accompanied by a decrease in volume can be enhanced by pressure [107];
- **Isostatic principle**: The transmittance of pressure is uniform and instantaneous [107].

As stated by Le Chatelier, HP affects any phenomenon (in food systems and others) where volume changes are involved, favoring reactions that cause decrease in volume, while reactions involving an increase of volume are inhibited [108, 109]. According to the principle of isostatic processing, presented in Figure 5, HP treatments are independent of product size and geometry, and their effect is uniform and instantaneous [110-113]. During the treatment the product is compressed by uniform pressure from every direction and then returns to its original shape when the pressure is released [114].

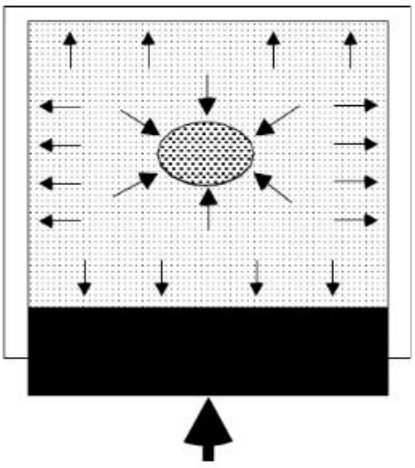

Figure 5. The principle of isostatic processing [106].

3.1.Effects of High Pressure on Living Organisms

HP exerts many effects on living organisms, affecting not only cell structural organization but also its metabolic processes [105]. In general, all pressure effects arise from a single influence, which corresponds to the volume reduction of the biological system, favoring the acquisition of more compact structural forms. Besides the structural alterations in biomolecules, pressure also disturbs the equilibrium of (bio)chemical reactions [115]. In Figure 6 are represented examples of some of the main effects of high hydrostatic pressure on cells and cellular components.

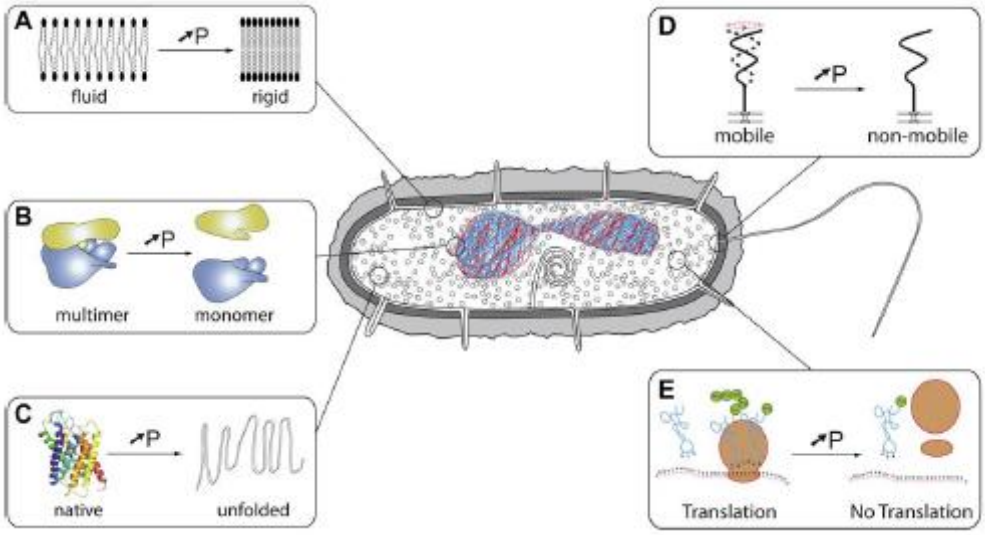

Figure 6. Examples of the effects of high hydrostatic pressure on cells and cellular components. A: lipids in membranes; B: multimeric protein assemblages. C: protein structure; D: cellular motility; E: protein translation by ribosomes [116].

Concerning the HP effects on lipid membranes, it is known that these structures are particularly pressure sensitive, because of its high compressible potential. With increasing pressure, lipid bilayers lose fluidity and became rapidly impermeable to water and other molecules, while protein-lipid interactions essential to the optimal function of the membrane are weakened [117]. Beyond changes in fluidity, HP also changes the composition of lipid membrane, through the increase of unsaturated fatty acids content, which require less carbon and energy to produce a similar effect on membrane fluidity than do saturated fatty acids. [118].

Hydrostatic pressure can disrupt non-covalent “weak” chemical bonds, which are essential to maintain protein structure and function. These changes are sufficient to affect multimer association and stability, as well as catalytic sites. In consequence, both

protein structure and function may be altered upon compression [119-122]. However, irreversible denaturation of proteins in aqueous solution usually requires pressures higher than 300 MPa.

As pressure leads to multimer disintegration, ribosomes are highly affected by HP, which causes its dissociation (70S→30S + 50S). In fact, subunit dissociation of the ribosomes seems to be one of the major factors of the cell death by HP, since bacterial cells only survive until the number of functional ribosomes decreases below a threshold level [123].

The application of HP may also cause changes in DNA structure and function. With the increasing pressure the DNA molecule is stabilized, and the double to single strand transition necessary for cell processes (such as replication, transcription and translation) may become more difficult, because of the transition temperature increase [124].

In addition, some other cellular changes can occur when the organisms are exposed to HP, such as loss of flagellar motility and alterations in cellular architecture [116]. Cell division is also indirectly influenced by pressure, because the activity of several division proteins should be possible targets of HP [105, 125-129]. The damage magnitude depends on the varying degree of tolerance of the organisms, the extent and duration of pressure and other environmental parameters, and in some critical cases these effects can result in cell death. Surprisingly, some living organisms are able to withstand such hostile environments despite the strong effect of hydrostatic pressure on cell structures and their functions [130]. They are known as piezophilic or piezotolerant organisms.

3.2.Practical Applications

3.2.1. Applications in Food Industry

HP processing technology has recently received considerable attention among food researchers [131]. This technology has traditionally been employed in areas different from that of foods, as in the case of ceramics, steel and super alloy production, extrusion, and synthetic materials [132]. Research regarding the effects of HP on foods and microorganisms was first begun in 1889 by Hite [133], at West Virginia University in the USA. In 1990, Meidi-ya Food Co. (Osaka, Japan) introduced to the market the first food products processed using only HP [134, 135]. Some of the foods processed by

HP currently commercialized are jams, fruit juice [136], meat, oysters, ham, fruit jellies and pourable salad dressings, salsa and poultry, beyond others [137]. Figure 7 shows several groups of commercialized food products processed by HP technology, and the number of HP-equipments operating between 1990 and 2011.

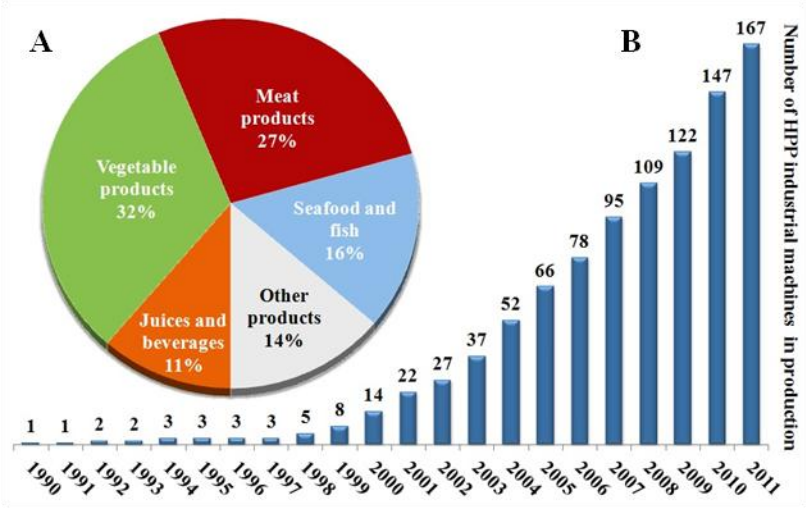

Figure 7. Distribution of food products currently processed by HP (A), and evolution of the number of HP industrial machines in production between 1990 and 2011 (B).

It is possible to conclude that the utilization of this technology in industry is growing over the years, in part as a result of the increasing consumer demand for minimally processed, additive-free and shelf-stable products. These consumer trends prompted food scientists to explore other preservation methods as alternative to traditional treatments, which rely on heating or cooling operations and may contribute to the degradation of various food quality attributes [138]. On the other hand, HP processing (as well as other non-thermal processing techniques) has the ability to destroy pathogenic microorganisms with minimal treatment yielding almost complete retention of nutritional and sensory characteristics of fresh foods without sacrificing shelf-life [139].

Currently, the widest application of HP processes within the food industry is mainly for extending the shelf-life of food products, although as research progresses other uses are foreseen. These include solute diffusion processes (salting, sugaring), assisted freezing-thawing processes, modification of functional properties of proteins and other macromolecules and the extraction of some cellular compounds [140-142].

3.2.2. Sub-Lethal High Pressure Stress Response

While inactivation of microorganisms by lethal HP is well investigated, the use of sub-lethal HP to cause a specific stress response in microorganisms is a less understood field and it can bring numerous interesting applications in biosciences [103, 143]. For instance, the modulation of microbial metabolic pathways as a response to different pressure conditions may lead to the production of novel compounds with potential use in industry. Therefore, studies in this context intend to obtain microorganisms with new metabolic or physiological characteristics, instead of its elimination (as observed in more traditional applications).

Bothun *et al.* (2004) [144] analyzed the behavior of a continuous culture of *Clostridium thermocellum*, a thermophilic bacterium capable of producing ethanol from cellulosic material, when exposed to HP (7.0 MPa, 17.3 MPa). The results of this study indicate that cell growth was inhibited by approximately 40% and 60% for incubations at 7.0 MPa and 17.3 MPa, respectively, relative to culture at atmospheric pressure. However, the authors also observed a shift in product selectivity (Figure 8) from acetate to ethanol when the culture was exposed to pressure. In fact, at HP, ethanol:acetate ratios increased $>10^2$ relative to atmospheric pressure, which may show a great interest in industrial bioethanol production.

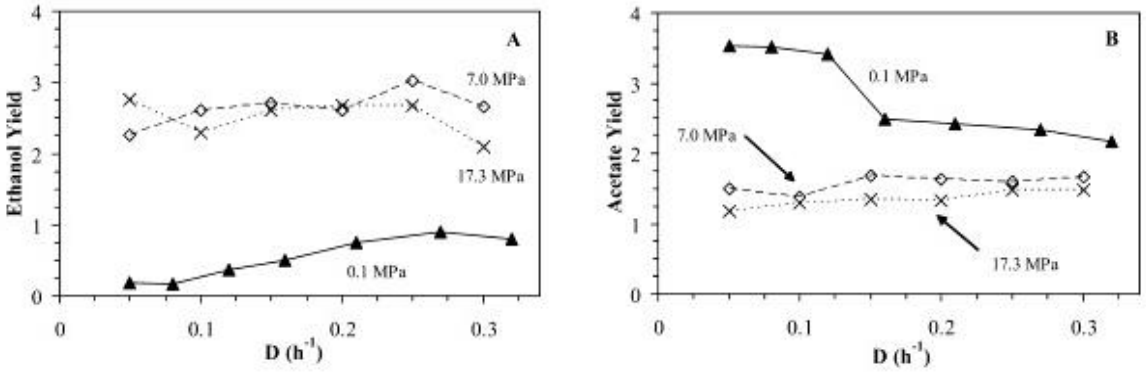

Figure 8. Ethanol (A) and acetate (B) yield in continuous cultures of *C. thermocellum* at a hydrostatic pressure of 0.1 MPa (*triangles*), 7.0 MPa (*diamonds*) and 17.3 MPa (*crosses*) [144].

There are only a few studies focusing on the influence of HP on fermentation processes. Picard *et al*. (2007) [145] monitored *in situ* alcoholic fermentation as one aspect of energetic metabolism by the yeast *Saccharomyces cerevisiae* under HP. The results showed that fermentation proceeds faster at low pressure (up to 10 MPa) than at

atmospheric pressure (Figure 9). Several phenomena could account for this increased activity under pressure, like the enhancement of the uptake of glucose in yeast at 10 MPa. At higher pressures, they become progressively repressed and they are completely inhibited above 87 MPa. This study showed that sub-lethal HP could enhance the microbial fermentative potential [145].

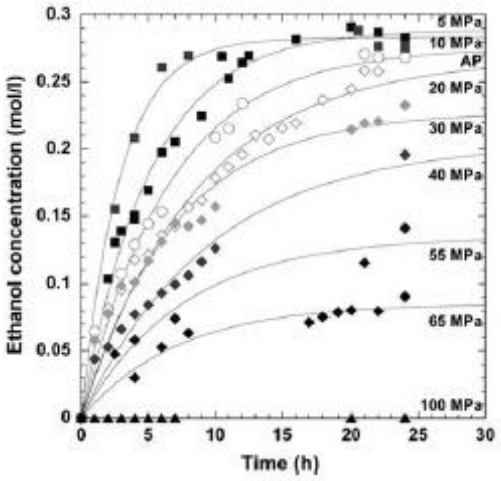

Figure 9. Kinetic of ethanol production as a function of pressure to 100 MPa [145].

In the last years, the concept of sub-lethal HP is gaining relevance and several studies in literature report the finding of microorganisms with acquired adaptation/resistance to pressure, envisaging new possible biotechnological applications. The main scientific findings about this subject are reviewed on a paper [146].

3.3.High Pressure Applied on Yogurt

Two strategies have been used for improving yogurt quality and preservation by application of HP: yogurt prepared from HP-treated milk; and HP processing (cold-pasteurization) of yogurt [147]. The studies performed using both approaches are described below.

3.3.1. Preparation of Yogurt from High Pressure-Treated Milk

Most published studies on the subject focus on this first methodology and, in general, the obtained results show that HP improves acid coagulation of milk without detrimental effects on important quality characteristics, such as taste, flavor, vitamins, and nutrients [148, 149].

One of the problems that yogurt producers must face, particularly in the case of low-fat yogurt, is to obtain a proper texture. Several studies report that the application of HP in the pre-treatment of cow milk improved the quality of yogurt in terms of its preservation and rheological properties [150, 151]. For instance, Needs *et al.* (2000) [152] recorded higher values of fracture stress in set yogurts made from pressure treated milk (60 MPa, 15 min) compared to heat treated milk, which translates in yogurts with a thick creamy consistency.

The combined effect of HP and thermal treatments has also been studied. Harte *et al*. (2003) [149] reported that yogurt made from milk subjected to HP (400–500 MPa) and thermal treatment (85^{o}C for 30 min) showed increased yield stress, elastic modulus and resistance to normal penetration, while having reduced syneresis, compared to yogurts made from thermally treated milk and from raw milk. Penna *et al.* (2007) [131] analyzed the effect of milk processing on the microstructure of probiotic low-fat yogurt and detected significant differences accordingly to the respective milk processing method applied. The authors concluded that the combined effect of HP and heat milk treatments led to compact yogurt gels with an uniform consistent microstructure with less physical defects, resulting in improved gel texture and viscosity [131].

3.3.2. High Pressure-Treated Yogurt

The other strategy used for improving yogurt quality consists in HP processing of yogurt, as a method of cold-pasteurization, allowing the inactivation of pathogenic and spoilage microorganisms. In this case, the main goal was to use HP as an alternative to the use of additives, which can adversely affect the yogurt taste, flavor, aroma and mouth-feel [153]. Jankowska *et al.* (2012) [154] submitted probiotic yogurt to different HP treatments and concluded that it is possible to apply pressures of 200 and 250 MPa to extend the durability as well as to improve the organoleptic properties of yogurt supplemented with probiotic bacteria.

Pressurized yogurt exhibited, in addition to higher amino acid content, higher viscosity and consequently an improved body and texture. In fact, it was proposed the treatment of yogurt at pressures ranging between 100 and 400 MPa to improve the texture of low-fat yogurts [155]. However, it is important to note that pressures exceeding 500 MPa significantly worsened the yogurt consistency [156].

Shah *et al.* [157] analyzed the effects of a HP treatment of 480 MPa on yogurt bacterial strains and it was concluded that viability of LAB was highly affected by this

level of pressure. Although the resistance to HP varies from strain to strain, it was verified that *L. bulgaricus* had the greatest sensitivity to a HP treatment of 400 MPa, whereas *S. thermophilus* showed the greatest resistance to the same treatment [158].

It is important to note that in all abovementioned studies the HP treatment was performed previously or after the fermentation process, aiming the improvement of yogurt textural properties and/or the extension of the shelf-life of the product. In any case lactic acid fermentation was performed under HP conditions, which show the great window of opportunity explored in this work.

II. DEFINING THE PROBLEM AND AIMS

As previously stated, until now few studies have been made about microbial growth under sub-lethal HP [146]. According to Picard *et al*. (2007) [145], pressures in the range of 5-10 MPa increase the alcoholic fermentation rate and yield of *S. cerevisiae*, comparatively to the same process at atmospheric pressure. Currently there are no published studies concerning the effect of HP on yogurt production process and it is expected that pressure will affect not only the fermentation rate, but also the organoleptic properties of the final product, allowing the discovery and possible development of products with novel characteristics.

Considering pressure as an extreme life condition, this study intends to: i) evaluate the potential of the application of HP technology in the yogurt production process; ii) use this work as a case-study of the effect of HP in microorganisms development, and *latusensu* in organisms in general.

The main goal of this study consists in the analysis of the effect of HP on yogurt production, namely on the fermentation rate.

To achieve the above-named purposes, several combinations of pressure/time must be tested, to identify the conditions that might result in some improvement on the fermentation process and/or in the achievement of a final product with possible different characteristics. Then, it is important not only to monitor lactic acid fermentation, but also to evaluate the effect of HP on yogurt main chemical composition, to better understand the implications of this treatment on the characteristics of the final product. In the view of this, pH, titratable acidity, reducing sugars, D-glucose, L-/D-lactic acid, acetaldehyde and ethanol were quantified. In addition, microbiological counts of *L. bulgaricus*, *S. thermophilus* and *B. lactis* were performed.

III. MATERIAL AND METHODS

1. Production of Probiotic Yogurt

1.1.Sample Preparation

UHT (Ultra High Temperature) treated semi-skimmed milk from Auchan was inoculated with plain probiotic yogurt Activia®, which is supplement with *Bifidobacterium animalis* subsp. *lactis* (DN-173 010 strain). The inoculum was added in the proportion of 80 mg of yogurt per mL of milk. The mixture was homogenized and then transferred to a heat sealed plastic bag (8 cm x 2.5 cm), designed to withstand HP conditions. All these steps were performed in an aseptic environment, within a laminar flow cabinet, to avoid sample contamination.

1.2.Fermentation

1.2.1. Fermentation under HP

Fermentation was carried at 43^{o}C, the process optimal temperature, under the different HP conditions tested in this work (5, 15, 30, 50 and 100 MPa). These experiments were conducted in High Pressure System U33, Unipress Equipment, Poland, own by the Chemistry Department of University of Aveiro. This equipment has a pressure vessel of 35 mm diameter and 100 mm height surrounded by an external jacket, connected to a thermostatic bath to control the temperature, using a mixture of propylene glycol and water as pressurizing fluid and to control the temperature in the external jacket. To use as control, fermentation was also performed under 0.1 MPa (atmospheric pressure), keeping all other parameters constant. Several samples were collected over the fermentation time and each experiment was run at duplicate, while the analyses were carried out in triplicate. Fermentation was arrested by immersion in an ice bath or in liquid nitrogen, in case of samples used for microbiological or for physicochemical analysis, respectively. In this last case, the samples were stored at -80^{o}C until its posterior analysis.

1.2.2. Fermentation under Combined Pressure Conditions

In parallel, a different type of experiments was carried out, in which the fermentation was performed at atmospheric pressure (0.1 MPa) after a pre-treatment of different pressure/time conditions, at the constant temperature of 43^{o}C. For that purpose, samples were initially exposed to a HP pre-treatment (50 or 100 MPa) during 90 or 180 minutes, and then transferred to a bath where the remaining fermentation was carried at atmospheric pressure. The collected samples were handled and stored in accordance with the described in the previous section ("Fermentation under HP").

Table 1. Experimental design of experiments performed at combined pressure conditions.

HP pre-treatment		**Fermentation at P_{atm}**
HP intensity (MPa)	**Time at HP (minutes)**	**Time (minutes)**
50	90	0
		270
100	90	0
		90
		210
		510
		600
100	180	0
		90
		210
		360
		510
		600

2. Characterization of Yogurt Preparations

2.1.Physicochemical Analysis

2.1.1. pH and Titratable Acidity

One of the main changes verified during the yogurt fermentation is the lactic acid production, which causes a decrease of pH over the time. In addition, the pH value is an easy-to-measure parameter, important to monitor the evolution of the fermentation process. In this work, pH of the fermentative medium was measured using a properly calibrated glass electrode (pH electrode 50 14, Crison Instruments, S. A., Spain), at 25^{o}C.

It is also important to determine titratable acidity as a monitoring parameter of lactic acid fermentation. Titratable acidity allows the calculation of the total acid content in each sample, through its acid-base titration, and the results are expressed as lactic acid concentration. The analysis was performed using a Titromatic 1S (Crison Instruments, S. A., Spain), accordingly to Chandan [159] with some modifications: 1.50 mL of yogurt sample were diluted in 10.5 mL of water and then titrated with a 0.1N NaOH solution, until pH of 8.9. The obtained results are expressed in g of lactic acid/L of yogurt.

2.1.2. Reducing Sugars Concentration

The concentration of reducing sugars over time provides the substrate consumption rate during fermentation. In this case, the main reducing sugars present in the sample are lactose, glucose and galactose, which are metabolized by the starter and probiotic strains over the fermentation time, leading to the production of lactic acid and other products.

To determinate the concentration of reducing sugars it was applied a colorimetric method using 3,5-dinitrosalicylic acid reagent (DNS), described by Miller, 1959 [160]. In this method, DNS reagent is an alkaline solution and reducing sugars (with a free aldehyde or ketone group) are able to reduce the 3-5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid, while the aldehyde group is oxidized to an aldonic acid, as represented in Figure 10. The produced acid has an orange color and therefore the color intensity of the solution depends on its reducing sugars concentration [161].

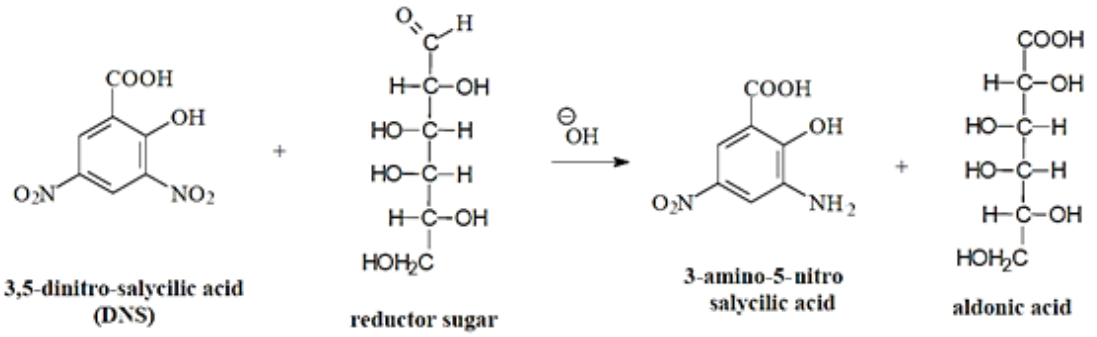

Figure 10. Reaction of reducing sugar with 3,5-dinitro-salycilic acid (DNS) reagent [161].

For that purpose, 1.0 mL of DNS reagent (which preparation is described in Appendix I) was added to 1.0 mL of sample and then the mixture was placed in a boiling water bath during 5 minutes. After that time, the mixture was cooled in an ice bath (to stop the reaction), diluted to 10 mL and then the absorbance was measured at 540 nm, in Multiskan GO Microplate Spectrophotometer (Thermo Scientific, Thermo Fisher Scientific Inc., USA). The concentration values were calculated using a calibration curve, obtained from glucose standard solutions, and are expressed in g of reducing sugars/L of yogurt.

2.1.3. D-Glucose Concentration

Despite of its relatively low concentration in milk, glucose is one of the main substrates involved in lactic acid fermentation. As previously referred, D-lactose is the most abundant sugar in milk and suffers hydrolysis prior to the fermentative process, originating D-glucose and D-galactose. Since many starter and probiotic strains are not capable of galactose digestion, only glucose is used as substrate for fermentation.

In this work D-glucose was measured using the enzymatic test kit D-Glucose GOD-POD (AK00161) from NZYTech, Lda. – Genes and Enzymes, Portugal, accordingly to the manufacturer's instructions and adapted for use in 96-well microplates. The principle of this method is described by the following reactions [162]:

$$\text{D-glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{D-gluconate} + H_2O_2$$

$$2\ H_2O_2 + \textit{p}\text{-hydroxybenzoic acid} + \text{4-aminoantipirine} \xrightarrow{\text{POD}} \text{quinoneimine dye} + 4\ H_2O$$

D-glucose is oxidized by glucose oxidase (GOD), producing D-gluconate and hydrogen peroxide. In the presence of peroxidase (POD) hydrogen peroxide is then oxidatively coupled with 4-aminoantipirine (4-AAP) and a phenolic compound (in this case *p*-hydroxybenzoic acid) to yield a red quinoeimine dye, with a maximal absorbance at 510 nm. The absorbance at 510 nm is quantitatively proportional to the concentration of glucose present in the sample [163].

To perform this analytical test, samples were centrifuged (10,000g for 15 minutes) and the obtained supernatant was collected and properly diluted to obtain D-glucose concentrations between 100 and 1000 mg/L. After the absorbance reading (λ=510 nm), D-glucose concentration was calculated using a calibration curve, taking into account the respective dilution, and the results were expressed in g of D-glucose/L of yogurt.

2.1.4. D-/L-Lactic Acid Concentration

During yogurt fermentation both lactic acid stereoisomers are produced, since *S. thermophillus* produces L-lactic acid, while *L. bulgaricus* synthesizes D-lactic acid. In consequence, the determination of the proportion between these two isomers can be used to the contribution of each starter to the fermentation process.

In this work, D- and L-lactic acid concentrations were determined with an enzymatic test kit D-/L-Lactic acid (AK00141) from NZYTech, Lda. – Genes and Enzymes, Portugal, accordingly to the manufacturer's instructions and adapted for use in 96-well microplates. With this test kit the assays for D-lactic and L-lactic acids are performed separately. The determination of D-lactic acid is based on the following two coupled reactions [164]:

$$\text{D-Lactate} + NAD^+ \xrightarrow{\text{D-LDH}} \text{Pyruvate} + NADH + H^+$$

$$\text{Pyruvate} + \text{D-Glutamate} \xrightarrow{\text{D-ALT}} \text{D-Alanine} + \text{2-Oxoglutarate}$$

The amount of NADH formed through the combined action of D-lactate dehydrogenase (D-LDH) and D-alanine aminotransferase (D-ALT) is measured at 340 nm. Since the first reaction is an equilibrium reaction, a coupled one is necessary, in order to complete the reaction [164].

The determine L-lactic acid requires a similar set of reactions but the oxidation to pyruvate by NAD^+ is catalyzed by L-lactate dehydrogenase (L-LDH) instead, as follows [164]:

$$\text{L-Lactate} + NAD^+ \xrightarrow{\text{L-LDH}} \text{Pyruvate} + NADH + H^+$$

$$\text{Pyruvate} + \text{D-Glutamate} \xrightarrow{\text{D-ALT}} \text{D-Alanine} + \text{2-Oxoglutarate}$$

Prior to the analysis, samples were submitted to a centrifugation (10,000g for 15 minutes) and the obtained supernatant was collected and properly diluted to obtain concentrations between 0.33 and 20 mg/L in the case of D-lactic acid and between 0.20 and 20 mg/L in the case of L-lactic acid. After the absorbance reading (λ=340 nm), concentration values were calculated using a calibration curve, taking into account the respective dilution, and the results were expressed in g of D- or L-lactic acid/L of yogurt.

2.1.5. Acetaldehyde Concentration

As mentioned above, acetaldehyde is a carbonyl compound formed by lactic acid bacteria during fermentation and corresponds to the main responsible by the typical yogurt flavor. Therefore, the measurement of acetaldehyde concentration in yogurt samples obtained by different fermentation conditions give us the potential influence of the pressure treatment on yogurt taste and flavor.

Acetaldehyde concentration was determined using the enzymatic test kit Acetaldehyde (AK00051) from NZYTech, Lda. – Genes and Enzymes, Portugal, accordingly to the manufacturer's instructions and adapted for use in 96-well microplates. The principle of this method is described by the following reaction [165]:

$$\text{Acetaldehyde} + NAD^+ + H_2O \xrightarrow{\text{Al-DH}} \text{Acetate} + NADH + H^+$$

The amount of NADH formed through the action of aldehyde dehydrogenase (Al-DH) can be detected at 340 nm and it is stoichiometric with the amount of acetaldehyde in the sample volume [165].

To perform this test, samples were previously centrifuged (10,000g for 15 minutes) and the obtained supernatant was collected and properly diluted to obtain

acetaldehyde concentrations between the linearity limits, 0.25 and 10 mg/L. After the absorbance reading (λ=340 nm), acetaldehyde concentration was calculated using a calibration curve, taking into account the respective dilution, and the results were expressed in mg of acetaldehyde/L of yogurt.

2.1.6. Ethanol Concentration

Ethanol concentration was also measured in this work, using an enzymatic test kit (AK00061) from NZYTech, Lda. – Genes and Enzymes, Portugal, accordingly to the manufacturer's instructions and adapted for use in 96-well microplates. Ethanol determination by this analytical test kit is based on the following reactions [166]:

$$\text{Ethanol} + NAD^+ \xrightarrow{\text{ADH}} \text{Acetaldehyde} + NADH + H^+$$

$$\text{Acetaldehyde} + NAD^+ + H_2O \xrightarrow{\text{Al-DH}} \text{Acetate} + NADH + H^+$$

The amount of NADH formed through the combined action of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (Al-DH), measured at 340 nm, is stoichiometric with twice the amount of ethanol in sample volume [166].

Before the analysis, samples were centrifuged (10,000g for 15 minutes) and the obtained supernatant was collected and properly diluted to obtain ethanol concentrations between the linearity limits, 0.13 and 6 mg/L. After the absorbance reading (λ=340 nm), ethanol concentration was calculated using a calibration curve, taking into account the respective dilution, and the results were expressed in g of ethanol/L of yogurt.

2.2.Microbiological Analysis

To study the effect of pressure on the viability of starter and probiotic strains, a microbial count was performed in samples fermented at different pressure conditions.

To perform the microbiological analysis, 1 g of probiotic yogurt sample was transferred aseptically into a sterile tube with 9 ml of Ringer's solution and homogenized. Each sample was prepared in duplicate. Then serial decimal dilutions in sterile Ringer's solution were prepared and 1 mL samples of the appropriate dilutions were spotted on the plates, also in duplicate. To all three quantified microorganisms, the enumeration was carried out using a pour plate technique, but different selective media and incubation conditions were used according to the microorganism in question. After

the incubation time, plates containing 15 to 300 colonies were enumerated, and the counts were expressed as $\log_{10}$ CFU/mL of probiotic yogurt.

2.2.1. *Lactobacillus bulgaricus* Count

The *L. bulgaricus* count was determined on double-layer agar plates of MRS (Lactobacillus Agar acc. de Man, Rogosa and Sharpe - Merck, Germany) medium, pH 5.7 ± 0.2, which was previously sterilized at 121°C for 15 minutes. The cultures were then enumerated after incubation at 30°C for 5 days [167].

2.2.2. *Streptococcus thermophillus* Count

The *S. thermophilus* count was carried out in M17 (Liofilchem, Italy) medium, pH = 7.2 ± 0.2, sterilized at 121°C for 15 minutes. The inoculations were incubated at 37°C for 72 h [156].

2.2.3. *Bifidobacterium lactis* Count

B. lactis count was performed accordingly to Darukaradhya *et al.*, 2006 [168], using the RCA (Reinforced Clostridial Agar – Liofilchem, Italy) medium, pH 6.8 ± 0.2. The agar medium was supplemented with aniline blue (0.3 g/L) and then sterilized at 115°C for 15 minutes. A dicloxacilin stock solution (0.2% w/v) was prepared, filter-sterilized using a 0.2 µm membrane (Cellulose Acetate 0.22 µm Syringe Filter, Frilabo, Portugal) and then added at a rate of 1 mL/L to the molten agar before pouring into plates. The plates were incubated anaerobically in gas jars using the Anaerocult® A system (Merck, Germany) for 72 h at 37°C prior to observation [168].

3. Activation Volumes Calculation

By definition, the activation volume (Va) corresponds to a quantity derived from the pressure dependence of the rate constant of a reaction [169] and its calculation is performed using Eq. 1:

$$\ln(k) = \ln(A) - Va \times \frac{p}{R_p \times T} \qquad (1)$$

where k is the reaction rate constant, A is a constant, Va the activation volume (cm^3/mol), p is the pressure (MPa), R_p the universal gas constant (8.314 cm^3 MPa /(K mol)) and T is the absolute temperature (K). The activation volumes were calculated by linear regression analysis.

4. Statistical Analysis

Differences between the results at different pressure conditions were tested at a 0.05 level of probability. The effects of pressure level were tested with a one-way analysis of variance (ANOVA), followed by a multiple comparisons test (Tukey HSD) to identify the differences between samples.

IV. RESULTS AND DISCUSSION

1. Effect of High Pressure on Physicochemical Parameters of Probiotic Yogurt

1.1.Monitoring of Fermentation under Different Pressure Conditions

In the initial stage of this work, inoculated milk samples were exposed to different pressure conditions (in the range of 0.1-100 MPa), at 43^{o}C, in order to study the effect of HP in lactic acid fermentation. As previously stated, the measuring of pH and titratable acidity as well as the determination of reducing sugars concentration correspond to the physicochemical parameters used in this work to monitor the extent of lactic acid fermentation. In all performed assays a control sample was also carried out at atmospheric pressure and the results presented in this section as "0.1 MPa" were obtained from the calculation of the mean of all scores. In order to understand if samples fermented at different pressure conditions have significant differences between them, a statistical analysis was carried out and the obtained results are presented in Appendix II – section a).

Figure 11 shows the pH variation of samples exposed to different pressure conditions over the fermentation time. In this case, it is possible to observe that the general tendency corresponds to a pH decrease over time, with exception of samples subjected to 100 MPa.

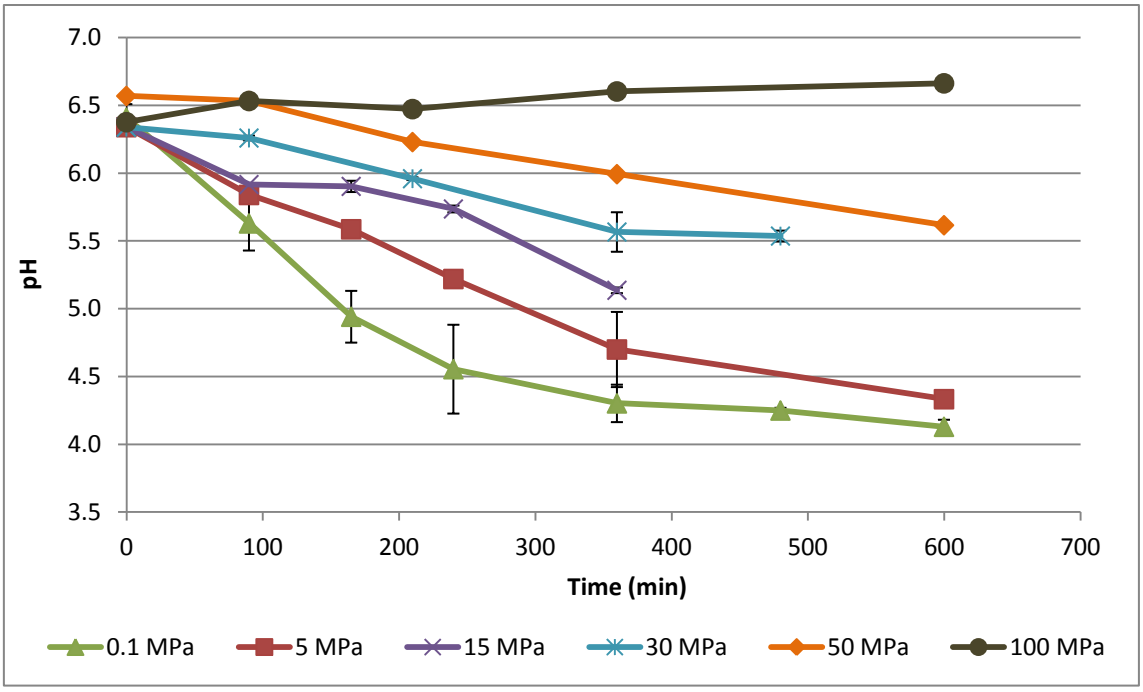

Figure 11. pH variation over the fermentation time, measured from samples exposed to different pressure conditions, in the range of 0.1-100 MPa.

At atmospheric pressure (0.1 MPa), a more accentuated pH reduction occurred in the first 180 minutes, after which the sample achieved a pH of 4.9. After this time the pH value continued to decrease and, at 360 minutes of fermentation, the pH variation has gradually begun to stabilize. It is important to note that the typical fermentation time applied in dairy industry to yogurt fermentation corresponds to 2-3 hours [4, 5], which is consistent with the time period with higher fermentation rate observed in the present work. When the fermentation was stopped (after 600 minutes), the obtained samples had a pH value of 4.1, which is lower than the pH referred in literature as the standard value required to yogurt (pH ≈ 4.5) [4]. In this case, the desired pH was reached approximately after 240 minutes of fermentation, a little further than the usual time used industrially.

Through the analysis of Figure 11 it is also possible to conclude that HP affects the pH variation over the fermentation time. With the increasing pressure it is possible to observe a lower pH variation and the pH value reached after 600 minutes is progressively higher, indicating that HP reduces the fermentation rate, which is more affected when the pressure intensity is higher. At 5 MPa the fermentation rate was lower than at atmospheric pressure and, in consequence, it was necessary more time to reach a pH of 4.5. However, at the end of fermentation (600 minutes) the analyzed samples

showed a pH value of 4.3, indicating that is possible to obtain yogurt fermented at these conditions. This goal becomes harder to achieve with the increasing pressure (due to the fermentation deceleration) and ultimately, at 100 MPa it was even observed a slight pH increase over time. Furthermore, it may be concluded that in these conditions the fermentation process have not occurred, judging by the almost constant pH value over time, which probably indicates that the bacterial and probiotic strains have been inhibited or destroyed by HP. For instance, it is known that some other bacterial strains (e.g. *Escherichia coli*) suffer inhibition of several important metabolic and physiological processes in the range of pressures evaluated in this work and may even lose its viability at 100 MPa [105].

The variation of titratable acidity over the fermentation time, at different pressure conditions, is presented in Figure 12.

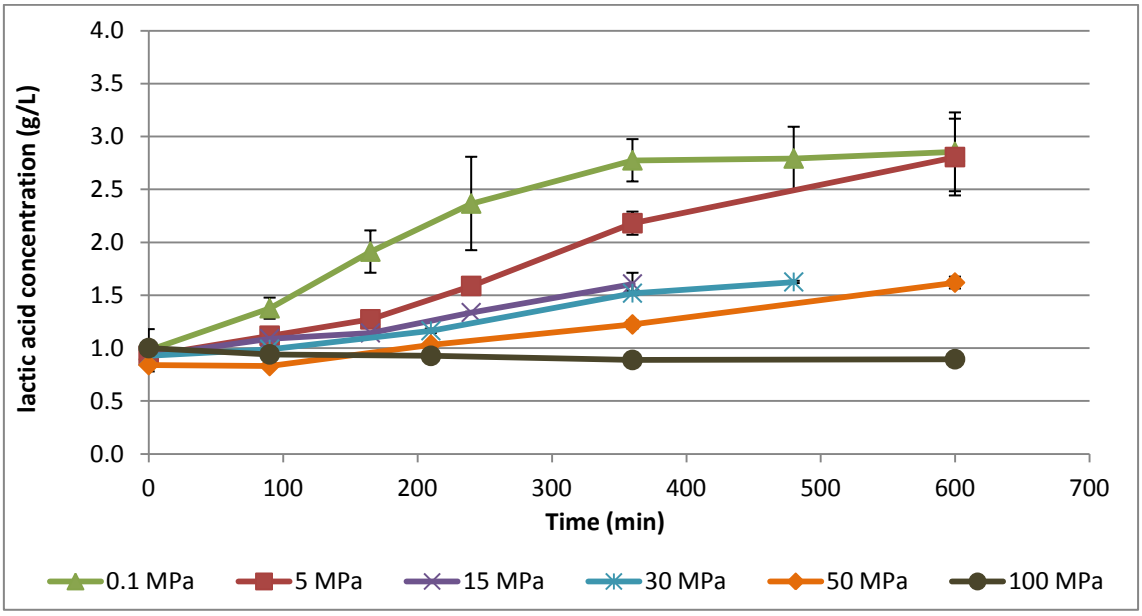

Figure 12. Variation of lactic acid concentration over the fermentation time, measured from samples exposed to different pressure conditions, in the range of 0.1-100 MPa.

The results obtained to this parameter are in accordance with the ones previously discussed for pH. In all pressures tested, with exception of 100 MPa, the acid concentration increased over time (provoking a pH decrease), due to acid formation as a consequence of LAB fermentative metabolism. Samples incubated at atmospheric pressure had a more marked increase in acid concentration during the first 360 minutes, varying from 1.0 to 2.8 g/L and, after that, the production of acid stabilized (2.9 g/L at

600 minutes). This behavior is similar to the observed to pH, which became constant after 360 minutes of fermentation at atmospheric pressure.

In what concerns to the results observed to different pressure conditions, it is possible to conclude that, with the increasing pressure, the variation of titratable acidity is less accentuated, which is also consistent with the pH results. Lactic acid fermentation is slowed down by HP and, in consequence, a lower quantity of acid accumulates in the fermentative medium, leading to the stabilization of pH.

During the first hours at 5 MPa, the fermentation was slower than at atmospheric pressure and, in consequence, the acid concentration was lower during this time. After 600 minutes of fermentation, the acid concentration seemed to reach similar values at both conditions (2.8 at 5 MPa; and 2.9 g/L at atmospheric pressure). Taking into consideration the results of the statistical analysis, it was observed that these samples (at 0.1 and 5 MPa) are not significantly different ($p > 0.05$), in terms of titratable acidity. Probably, it would be possible to detect a similar effect in samples exposed to 15 and 30 MPa, but the absence of results at 600 minutes do not allow to express this conclusion. When the process was carried out at 100 MPa, there was no variation detected in titratable acidity, since fermentation was probably interrupted at these conditions, as previously stated in this work.

The previously discussed results focus on the effect of HP on product formation during lactic acid fermentation, but it is also important to study its influence on substrate consumption. Figure 13 shows the reducing sugars concentration over the fermentation time, at different pressure conditions.

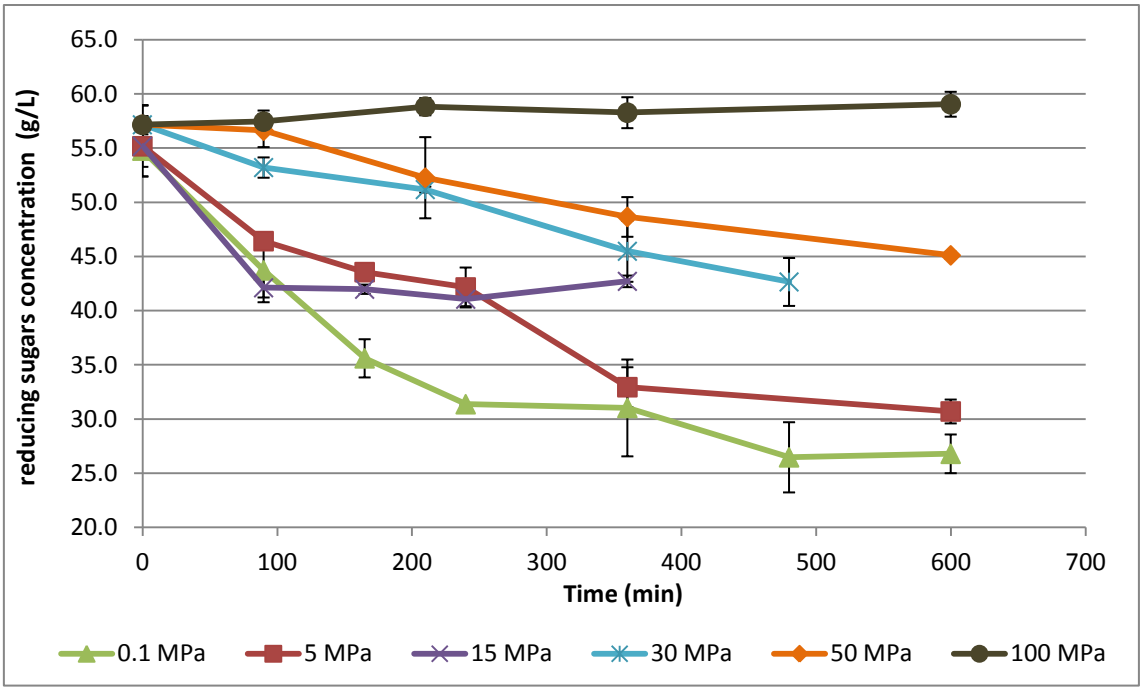

Figure 13. Variation of reducing sugars concentration over the fermentation time, measured from samples exposed to different pressure conditions, in the range of 0.1-100 MPa.

The results in Figure 13 are consistent with those observed to pH and titratable acidity. With the increasing pressure, the sugar consumption tended to decrease, suggesting, once more, that pressure leads to a slower fermentative metabolism. For instance, at 5 MPa, the variation in reducing sugars concentration over time was lower than at atmospheric pressure and, in consequence, the final products obtained at both conditions had significantly different sugar content ($p > 0.05$). Once again, at 100 MPa it was not registered any fermentative activity, since the reducing sugars concentration remained practically stable over time, indicating that starter bacteria were inhibited or destroyed by the prolonged time at such harsh conditions (this question is discussed further).

In order to better elucidate the effect of pressure on the fermentation process, the results at 90 and 360 minutes were plotted as a function of pressure (Figures 14, 15 and 16 for pH, titratable acidity and reducing sugars concentration, respectively).

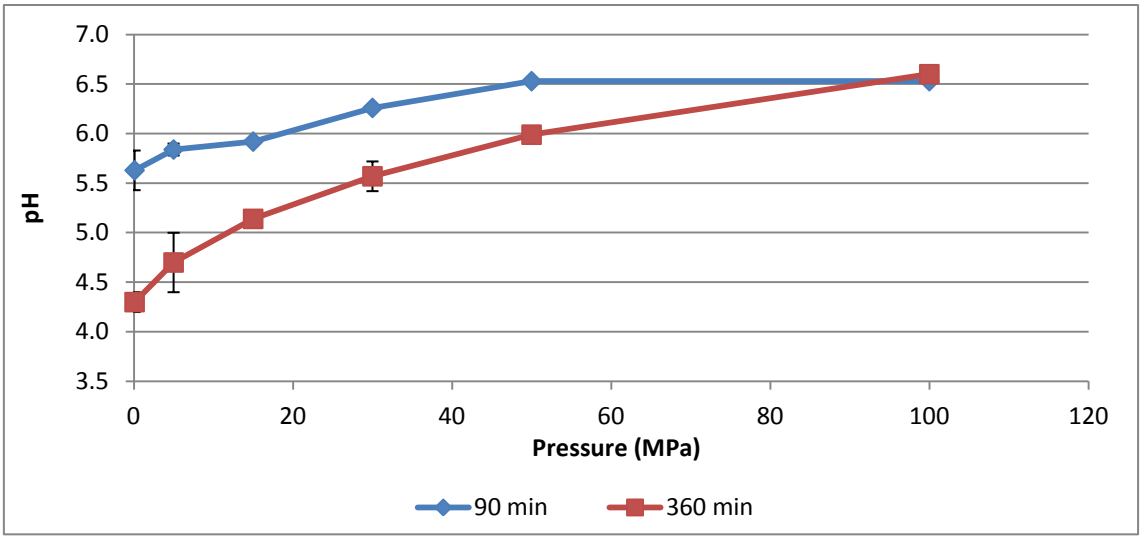

Figure 14. pH value after 90 and 360 minutes of fermentation, as a function of pressure.

In both cases it was observed an increase in pH with the increasing pressure, i.e. the higher the pressure, the higher the pH of the samples collected after 90 and 360 minutes of fermentation. These results are in accordance with the previously discussed: with the increasing pressure, the fermentative process is slower and, at the same fermentation time, the samples subjected to HP have a higher pH value (because of the lower content of acid formed). It is possible to note that at 90 minutes of fermentation the pH values are always higher than those at 360 minutes (with exception of the samples at 100 MPa), which makes sense considering that, after 90 minutes, fermentation is only just beginning and, as a consequence, the milk acidification is not very significant yet. At 100 MPa the pH value is very similar after 90 and 360 minutes of fermentation, since the samples at this pressure haven't suffered a noteworthy pH variation, due to the possible inhibition of the fermentation process.

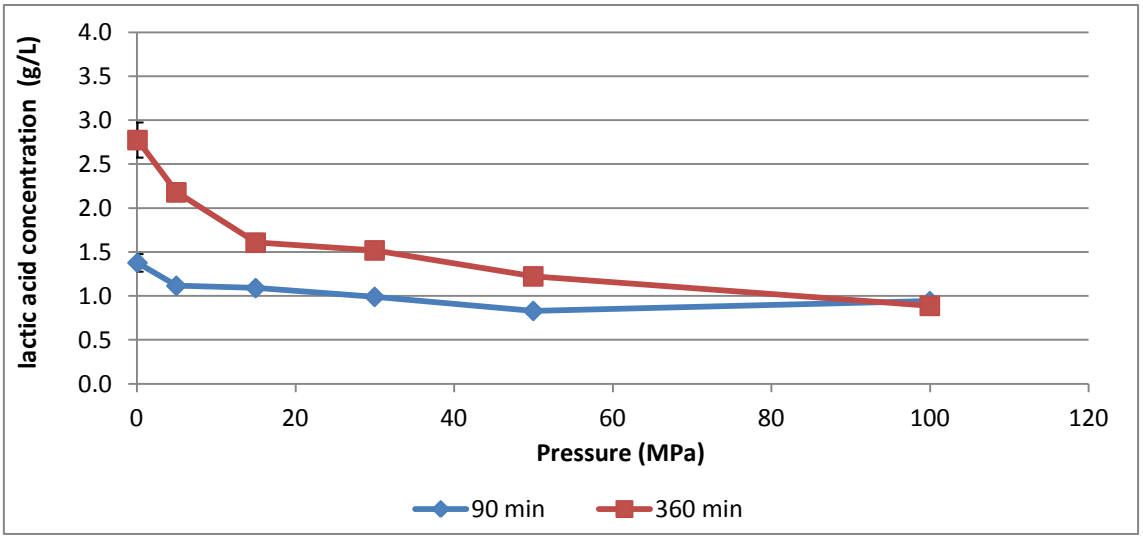

Figure 15. Lactic acid concentration, after 90 and 360 minutes of fermentation, as a function of pressure.

Regarding titratable acidity, the general tendency corresponds to a decrease of acid concentration with the increasing pressure, indicating that samples exposed to HP have a lower concentration of acid, due to the inhibitory effect of pressure on fermentation. Once more, at 100 MPa the acid concentration obtained at 90 and 360 minutes is approximately the same, because at these conditions the fermentative process (and then the acid production) is not very significant. In conclusion, the behavior in this case is similar to the previously observed to pH.

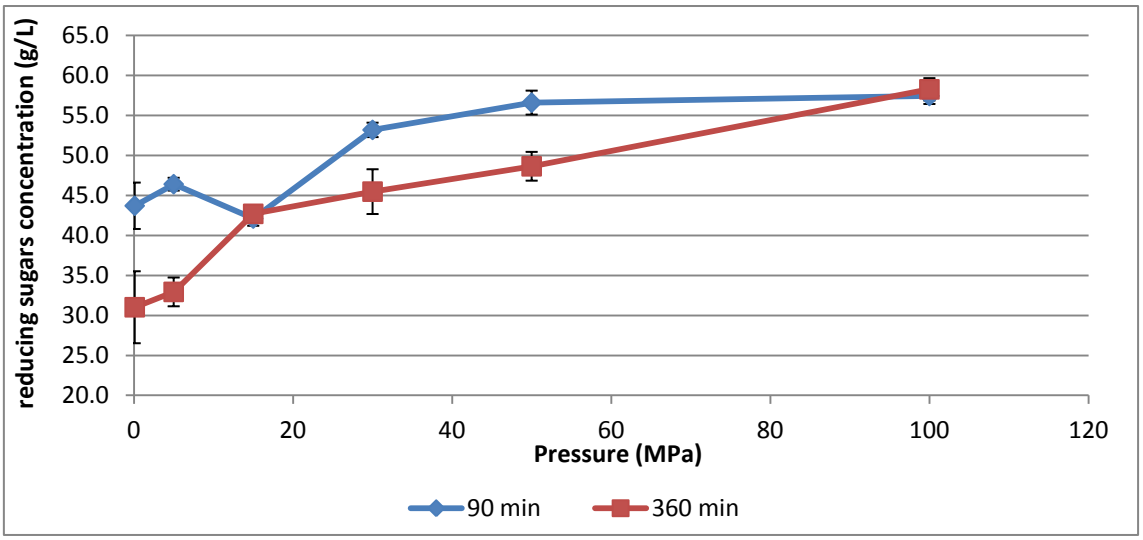

Figure 16. Reducing sugars concentration, after 90 and 360 minutes of fermentation, as a function of pressure.

It is important to note that the reducing sugars concentration value corresponding to 15 MPa seems incongruent and might be a consequence of an experimental error. Nevertheless, the results of Figure 16 are consistent with those observed to pH and titratable acidity: the reducing sugars concentration tended to increase with the increasing pressure, showing that samples under HP conditions have lower sugar/substrate consumption, due to the gradual fermentation inhibition.

In conclusion, pH, titratable acidity and reducing sugars concentration were clearly affected by pressure during lactic acid fermentation. Samples exposed to HP have shown lower substrate consumption and lower acid production, which indicates a progressive slowdown of the fermentative process caused by these conditions. This effect is probably a result of the loss of metabolic activity by the microbial strains (involved in lactic acid fermentation) when exposed to pressure, since it compromises several important cellular and physiological functions.

1.1.1. Activation Volumes Calculation

The Va value gives information about the effect of pressure on the reactions rate, i.e. if a reaction is accelerated or slowed down by pressure. When a positive Va is observed, the reaction is slowed down by pressure, and vice versa. In addition, the higher the Va, the higher the effect of pressure on the reaction.

In this section, the Va of fermentation was estimated using the variation of H^+ concentration, titratable acidity and reducing sugars concentration. The Va determination was based on the Eyring law, accordingly to what is accurately explained in Appendix III. Table 2 shows the Va values which resulted from these calculations. It must be emphasized that this kinetic analysis has never been performed neither to yogurt fermentation nor to fermentation with different microbial strains under pressure. Until now, only one work reported reaction rate constants under pressure, for ethanol formation, but no Va value was calculated.

Table 2. Activation volumes of fermentation, estimated to each physicochemical parameter.

Physicochemical Parameters	Activation Volumes (cm^3/mol)	r^2
H^+ concentration	54.1	0.94
Titratable acidity	37.4	0.81
Reducing sugars concentration	64.0	0.94

As shown in Table 2, the estimated Va values were positive for all three physicochemical parameters, indicating that the reactions involved in lactic acid fermentation are slowed down by pressure. It is important to note that the fermentation process is comprised by several biochemical reactions, thus the obtained Va values correspond to a global result, dependent of all different reactions involved.

Comparing the values exhibited in Table 2, it is possible to conclude that the Va estimated to reducing sugars concentration was the greatest of all three, showing that this parameter had the highest sensitivity to pressure. These results suggest that, during lactic acid fermentation, sugar consumption is more affected by pressure than product formation.

Concerning pH values, the estimated Va is higher than the calculated to the titratable acidity, indicating that pH is more affected by pressure than the acid concentration. It would be expected that the Va values were similar to both parameters, since usually there is a direct correlation between them. However, it was observed that pH is more sensitive to HP, i.e. under pressure conditions, the pH reduction is less accentuated (pH values vary less over the fermentation time), comparatively to what observed to titratable acidity. Therefore, it may be concluded that, under pressure, acid production is not always reflected in the pH value. This effect is probably due to differences in the bacterial metabolism caused by HP, which might change the proportions of organic acids (with different pKa values) produced during fermentation. The results also indicate that the production of stronger acids (with lower pKa, then with more influence in pH) might have been more affected by pressure than the production of weaker acids. It seems that, under HP, occurs a higher relative production of weaker acids. In consequence, pH suffers a lower variation over time, leading to a higher Va value. These results point, once more, to a possible effect of pressure on the metabolism of the fermentative bacteria, namely in what concerns to the formation of organic acids. In order to achieve a proper understanding of this effect, it would be necessary to perform a detailed analysis of the acid profile of samples fermented at different pressure conditions.

1.2.Fermentation under Combined Pressure Conditions

The assays discussed in this section were performed using combined pressure conditions, i.e. fermentation was carried out under HP during the first hours (“HP pre-

treatment") and then the sample was transferred to a bath, where the fermentation proceeded at atmospheric pressure. Different pre-treatments were tested, with variable intensity and duration, in order to determine its effect on the fermentative potential of the bacterial strains and on some other characteristics of the sample. Another purpose of the experiment was to establish if it would be possible to obtain yogurt after the pre-treatment with HP, or if it would be impossible to recover the initial fermentative potential. In addition, a statistical analysis was performed (wherever possible) to confirm if the differences between samples are significant. The results are expressed in Appendix II – section b).

1.2.1. Pre-treatment of 50 MPa for 90 minutes

In the first assay, a pre-treatment of 50 MPa for 90 minutes was tested, after which the samples were transferred to atmospheric pressure during 270 minutes. Figures 17 and 18 show the variation of pH and acid concentration, respectively, over the fermentation time, not only in pre-treated samples, but as well in samples fermented at atmospheric pressure and 50 MPa. The time correspondent to the pre-treatment (90 minutes) is represented as negative, to facilitate the further comparison between all the tested conditions.

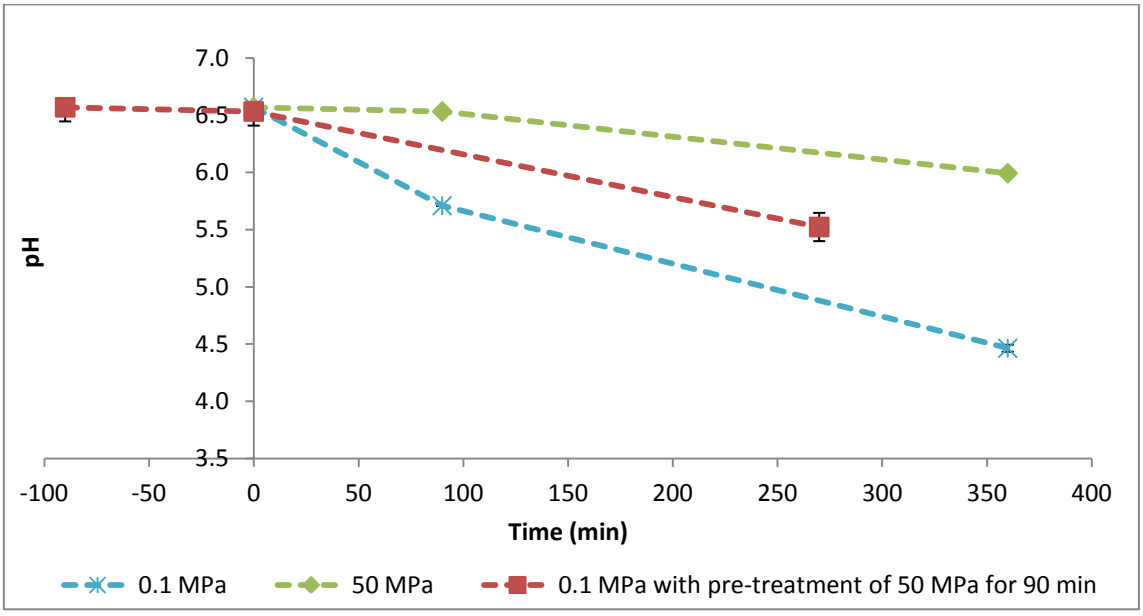

Figure 17. Variation of pH over the fermentation time in samples with pre-treatment of 50 MPa for 90 minutes and in samples fermented at 0.1 and 50 MPa.

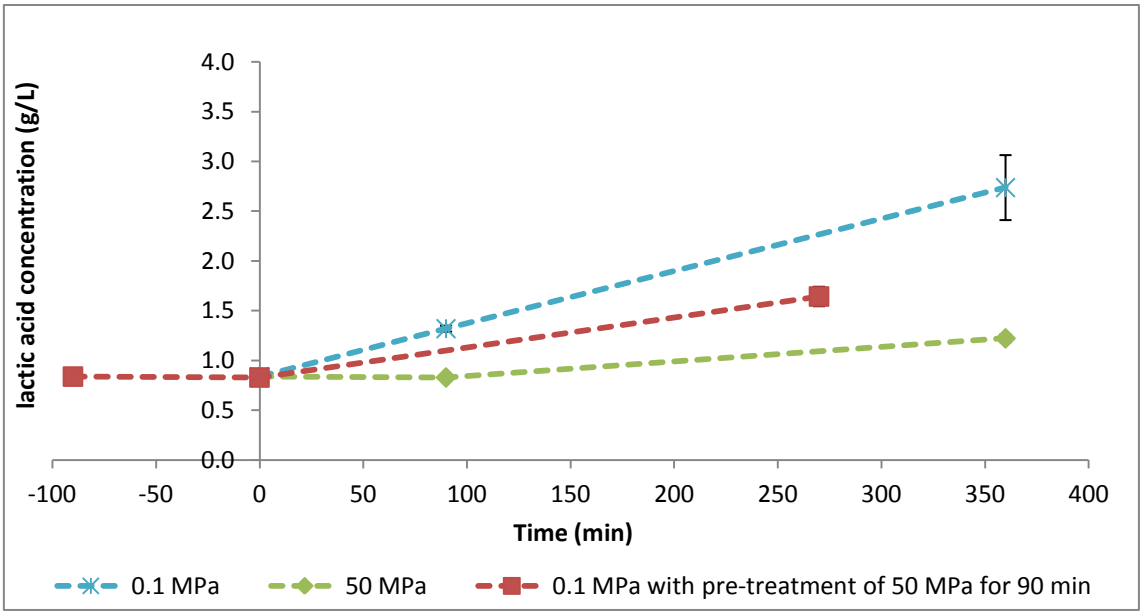

Figure 18. Variation of lactic acid concentration over the fermentation time in samples with pre-treatment of 50 MPa for 90 minutes and in samples fermented at 0.1 and 50 MPa.

During the pre-treatment time, at 50 MPa, pH and titratable acidity remained constant, indicating that, at these conditions, the fermentative activity did not occur. After 90 minutes of pre-treatment, the fermentation was then performed at atmospheric pressure and from that moment on it was observed an increase in acid concentration and a pH decrease, which indicates the occurrence of fermentative metabolism. However it is not possible to discuss quantitatively the exact fermentative behavior observed after the pre-treatment time, since there are not enough results describing the parameters over these periods (we only have results correspondent to the initial and final times). Figures 17 and 18 also indicate that, after the pre-treatment, the fermentation rate seems to be in between the observed to (untreated) samples at atmospheric pressure and samples at 50 MPa. These results may demonstrate that the pre-treatment has not destroyed the bacterial strains, but it has affected its metabolic activity, because then, when the conditions were "optimal", the fermentative bacteria could not recover completely from the pressure shock.

The variation of reducing sugars concentration over the fermentation time, not only in pre-treated samples, but as well in samples fermented at atmospheric pressure and 50 MPa, is represented in Figure 19.

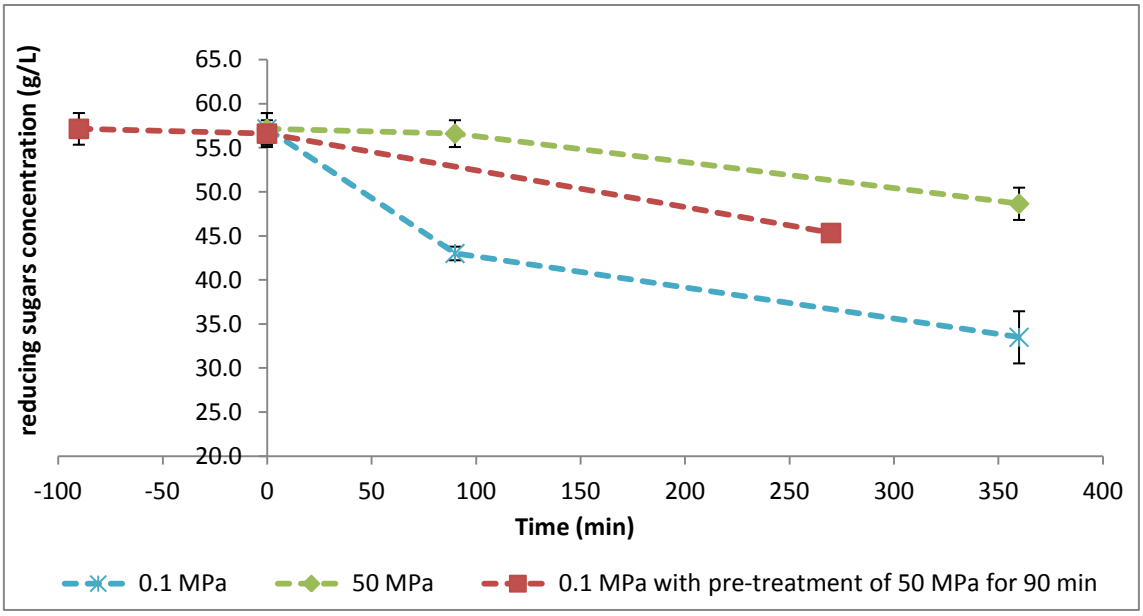

Figure 19. Variation of reducing sugars concentration over the fermentation time in samples with pre-treatment of 50 MPa for 90 minutes and in samples fermented at 0.1 and 50 MPa.

Figure 19 shows that there was no considerable variation in reducing sugars concentration during the pre-treatment at 50 MPa for 90 minutes. However, when the samples were transferred to atmospheric pressure, the sugar content seemed to decrease, possibly indicating that the fermentation process was taking place, since substrate was being consumed. In this case, the sugar consumption rate appears to be in between the observed to (untreated) samples at atmospheric pressure and samples at 50 MPa, which is in accordance with the previously discussed to pH and titratable acidity.

In conclusion, the pre-treatment at 50 MPa caused fermentative inhibition of the microbial strains, but not its complete destruction (since after that, at atmospheric pressure, it was possible to detect fermentative activity). However, this pre-treatment might have caused damages in cells, because the fermentative rate was not completely recovered.

1.2.2. Pre-treatment of 100 MPa for 90 minutes

A pre-treatment of 100 MPa for 90 minutes was also analyzed in this work. After that time under HP, the samples were then transferred to atmospheric pressure, where remained for 600 minutes. Figures 20 and 21 show the variation of pH and acid concentration, respectively, over the fermentation time, not only in pre-treated samples, but as well in samples fermented at atmospheric pressure and at 100 MPa. The time

correspondent to the pre-treatment (90 minutes) is represented as negative, to facilitate the further comparison between all the tested conditions.

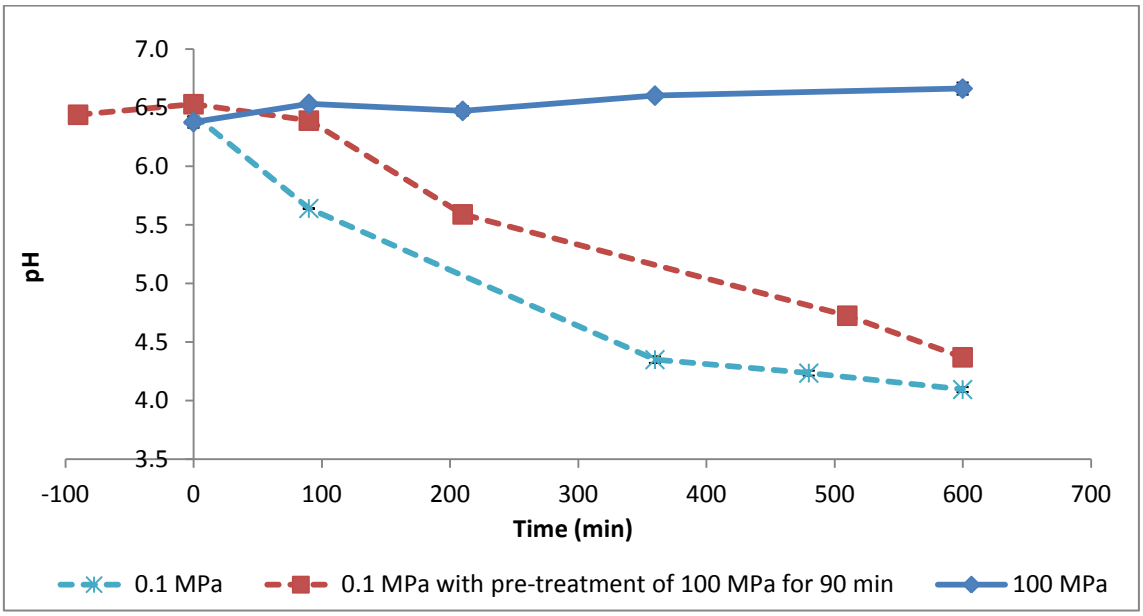

Figure 20. Variation of pH over the fermentation time in samples with pre-treatment of 100 MPa for 90 minutes and in samples fermented at 0.1 and 100 MPa.

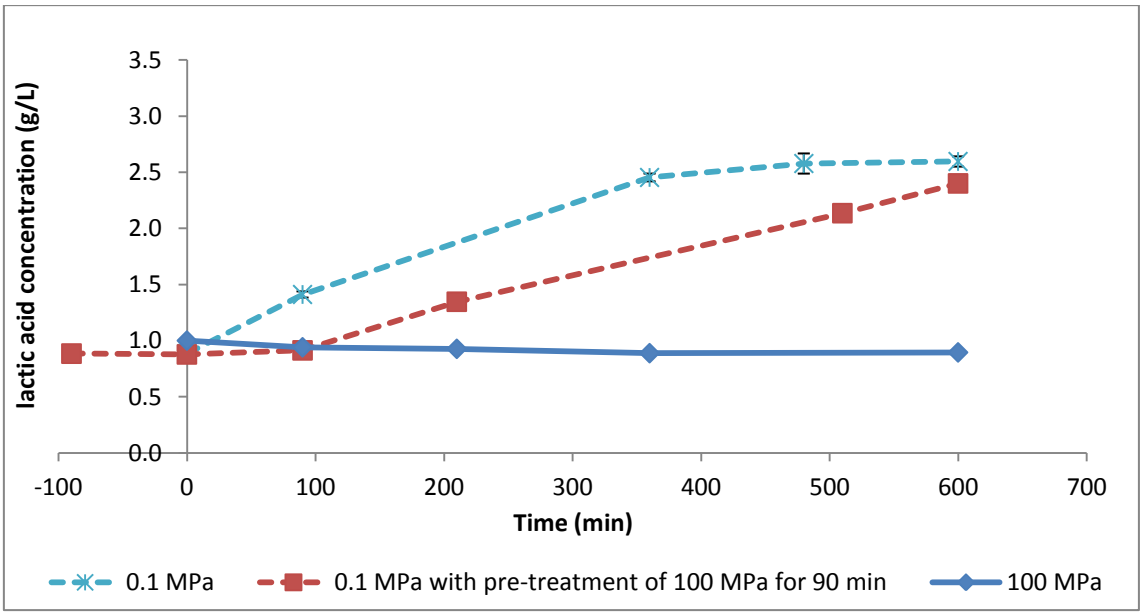

Figure 21. Variation of lactic acid concentration over the fermentation time in samples with pre-treatment of 100 MPa for 90 minutes and in samples fermented at 0.1 and 100 MPa.

During the pre-treatment of 100 MPa for 90 minutes, both pH and acid concentration remained constant, indicating that fermentative activity was not notable at this stage. These results are consistent with those observed at 100 MPa, since it was not detected any variation in pH and acid concentration at these conditions, as previously discussed. However, when the pre-treated samples were transferred to atmospheric pressure, the fermentative process initiated, judging by the increase in acid concentration (and the consequent pH decrease) over time. In general, the fermentation rate of pre-treated samples was lower than the observed in (untreated) samples at atmospheric pressure. After 600 minutes of fermentation at 0.1 MPa, pH and acid concentration values reached for both samples were very close, but significantly different ($p < 0.05$): pH of 4.4 and acid concentration of 2.4 g/L in the case of pre-treated samples; pH of 4.1 and acid concentration of 2.6 g/L in samples without pre-treatment. However, it is important to note that, in both cases, the final products reached the standard pH value required to produce yogurt (pH ≈ 4.5). It is also important to highlight that the bacterial strains survived the harsh conditions of pre-treatment and were able to decrease the pH and increase the titratable acidity to values in the range of those obtained at atmospheric pressure, in about the same fermentation period.

Figure 22 represents the variation of reducing sugars concentration over the fermentation time, not only in pre-treated samples, but as well in samples fermented at atmospheric pressure and 100 MPa.

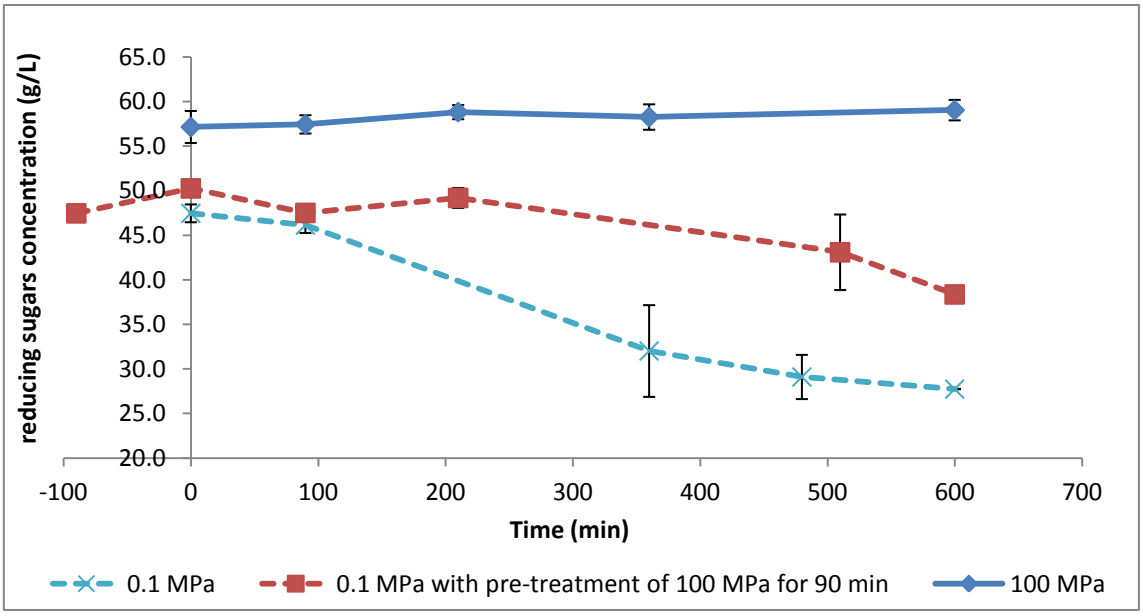

Figure 22. Variation of reducing sugars concentration over the fermentation time in samples with pre-treatment of 100 MPa for 90 minutes and in samples fermented 0.1 and 100 MPa.

It is possible to note, in Figure 22, a significant difference ($p < 0.05$) between the initial (0') values of reducing sugars concentration to different pressure conditions, but mostly in the case of 100 MPa. The results at 100 MPa were obtained in a different assay (using a different sample), which could explain this difference. However, it should be considered that the results at 100 MPa are exhibited only to assist the analysis and discussion, showing that, at these conditions, the sugar content remains practically constant over time.

During the 90 minutes of pre-treatment at 100 MPa, the reducing sugars concentration has shown a slight tendency to increase. However, after that time, the fermentation was performed at atmospheric pressure and, from that moment on, it has been observed a gradual decrease in reducing sugars content, slower in the first 210 minutes and then quicker until the end of the assay. These results show that substrate was being consumed and therefore fermentation was taking place at these conditions. After 600 minutes of fermentation, the pre-treated samples had a significantly lower ($p < 0.05$) sugar consumption over time, comparatively to samples at atmospheric pressure (without HP pre-treatment), indicating that the pre-treatment applied might have affected the fermentative potential of the bacterial strains.

In conclusion, during the pre-treatment at 100 MPa for 90 minutes, the starter strains have not been destroyed (at least in great extension), but its metabolic activity

suffered inhibition. After that, at atmospheric pressure, fermentation was initiated, but with a lower rate, since the sugar consumption and acid production were always lower than in untreated samples at atmospheric pressure.

1.2.3. Pre-treatment of 100 MPa for 180 minutes

In this last case, a pre-treatment of 100 MPa for 180 minutes was tested, after which the samples were transferred to atmospheric pressure during 600 minutes. Figures 23 and 24 show the variation of pH and acid concentration, respectively, over the fermentation time, not only in pre-treated samples, but as well in samples fermented at atmospheric pressure and 100 MPa. The time correspondent to the pre-treatment (180 minutes) is represented as negative, to facilitate the further comparison between all the tested conditions.

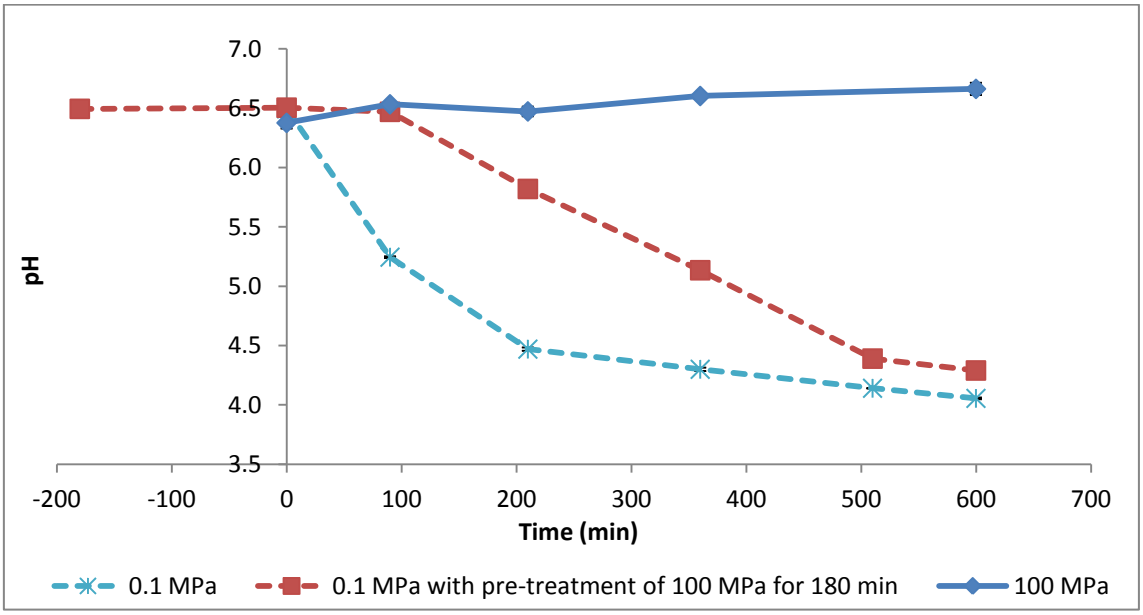

Figure 23. Variation of pH over the fermentation time in samples with pre-treatment of 100 MPa for 180 minutes and in samples fermented 0.1 and 100 MPa.

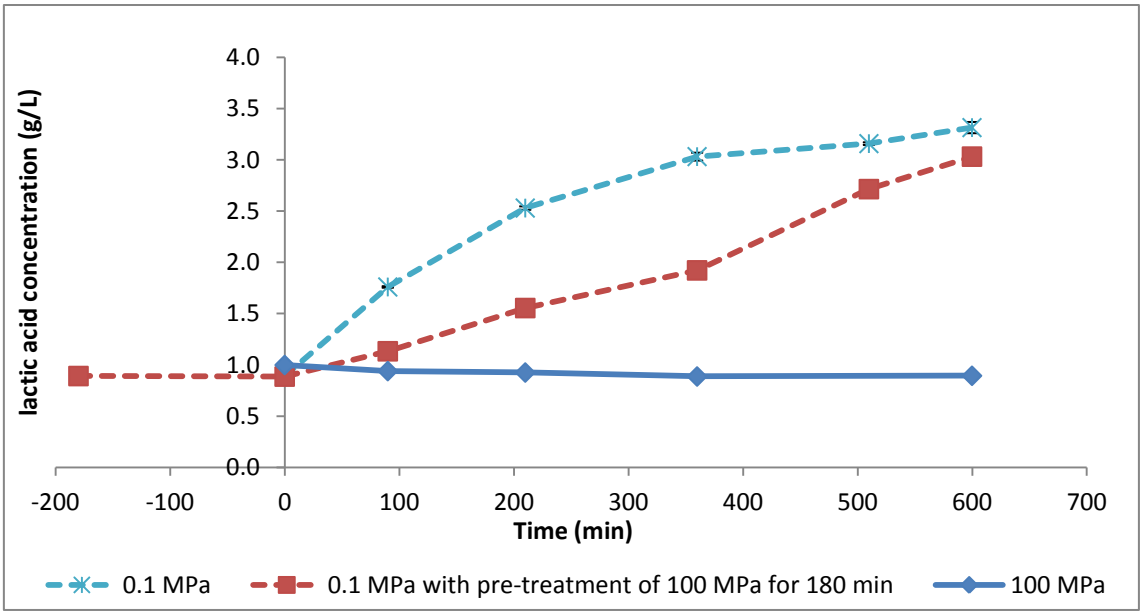

Figure 24. Variation of lactic acid concentration over the fermentation time in samples with pre-treatment of 100 MPa for 180 minutes and in samples fermented at 0.1 and 100 MPa.

Once more, during the pre-treatment time, both pH and acid concentration remained constant, indicating that fermentation was not occurring at these conditions. Then, when the samples were transferred to atmospheric pressure, pH started to decrease and titratable acidity started to increase, as a result of fermentative activity. Curiously, during the first 90 minutes at these conditions, pH variation was almost imperceptible, in contrast to what observed for titratable acidity.

Samples fermented at atmospheric pressure without HP pre-treatment showed a more marked pH decrease and acid concentration increase during the first hours of fermentation and, after that, the fermentation rate gradually decreased until the end of the process. The behavior was very different in the case of pre-treated samples, in which the fermentation rate at atmospheric pressure was relatively constant over time. In consequence, pH and acid concentration values, reached after 600 minutes of fermentation, were significantly different ($p < 0.05$) in samples with or without pre-treatment: pH of 4.3 and acid concentration of 3.0 g/L in the case of pre-treated samples; pH of 4.1 and acid concentration of 3.3 g/L in samples without pre-treatment. It is important to note that in both cases the pH required to obtain yogurt (≈ 4.5) was reached. These results indicate that the microbial strains have resisted the applied pre-treatment, promoting the development of a product with yogurt typical acidity and pH, with these values in the same range of the observed to untreated samples.

Figure 25 represents the variation of reducing sugars concentration over the fermentation time, not only in pre-treated samples, but as well in samples fermented at atmospheric pressure and 100 MPa.

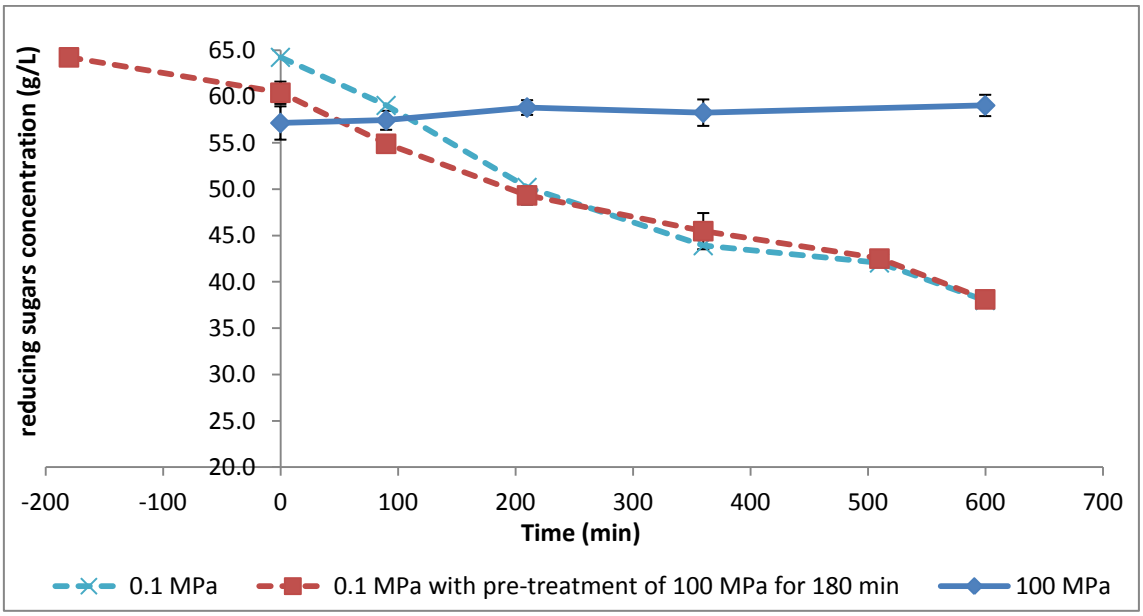

Figure 25. Variation of reducing sugars concentration over the fermentation time in samples with pre-treatment of 100 MPa for 180 minutes and in samples fermented at 0.1 and 100 MPa.

It is possible to observe, in Figure 25, that the reducing sugars concentration tends to decreases from 64.3 to 60.4 g/L during the 180 minutes of pre-treatment. These results are not in accordance with the results obtained in the previous section, which showed that samples exposed to 100 MPa had not relevant sugar consumption. Therefore, this difference may be attributed to a possible experimental error.

After the pre-treatment, when the samples were exposed to atmospheric pressure, the reducing sugars concentration gradually decreased over the fermentation time, which indicates substrate consumption by the bacterial strains. The fermentation rate seems to be similar in both samples at atmospheric pressure (with or without pre-treatment) and, after 600 minutes of fermentation, the reducing sugars concentration values were not significantly different in the two samples (38.1 and 38.0, in pre-treated samples and samples without pre-treatment, respectively; $p > 0.05$).

In conclusion, the results of this section suggest that during the pre-treatment at 100 MPa the fermentation process did not proceed. After this time, when the fermentation was carried out at "optimal" conditions (atmospheric pressure, 43^{o}C), it

has been detected substrate consumption and milk acidification, denoting that fermentation was taking place at these conditions. Moreover, pre-treated samples showed a sugar consumption similar to the observed at (untreated samples) at atmospheric pressure, even though acid production was usually lower.

1.3. Monitoring of Other Physicochemical Parameters under Different Pressure Conditions

To better understand the pressure effects on yogurt production, several other physicochemical parameters were assessed, using enzymatic test kits. For this purpose, specific pressure conditions were selected to perform the study: 5 MPa, since the previously discussed results shown the possibility to produce yogurt in this conditions; 100 MPa, because fermentation suffered inhibition; at 0.1 MPa with pre-treatment of 100 MPa for 180 minutes, since the fermentation rate was not very different from the observed at atmospheric pressure; and at last, at 0.1 MPa as control. The results of this section were also subjected to a statistical analysis, in order to verify if samples at different conditions have significant differences. The results are expressed in Appendix II – section c).

It is important to note that, to quantity all parameters bellow, the samples were centrifuged and the supernatant was collected prior to the analysis. Therefore, the presented results refer to product concentration in the supernatant.

1.3.1. D-Glucose Concentration

D-Glucose corresponds to one of the parameters analyzed in this section and the obtained results are represented in Figure 26.

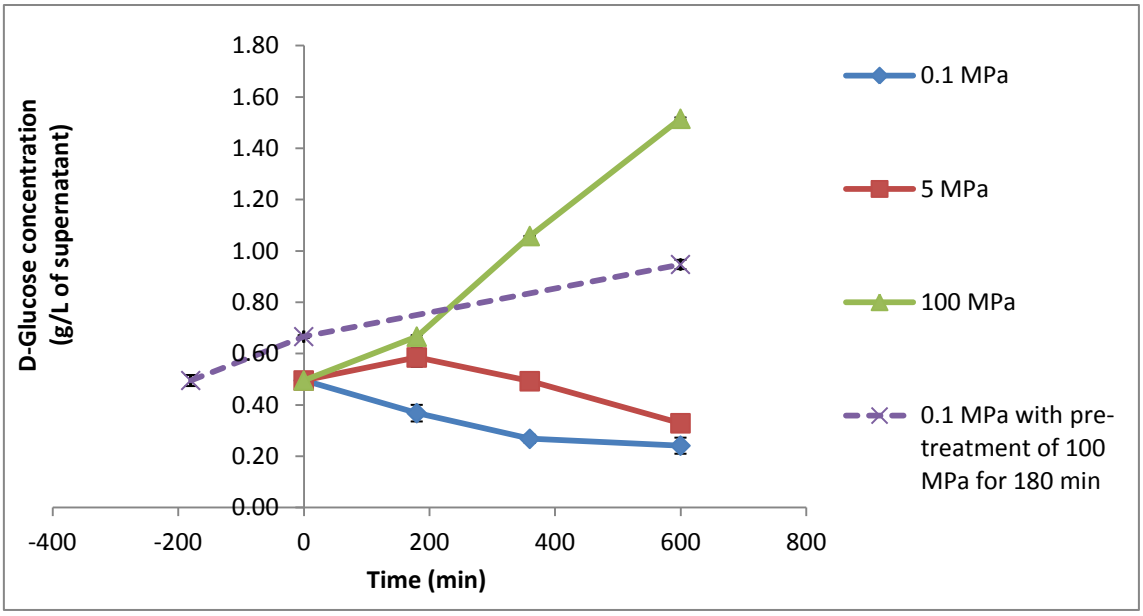

Figure 26. Variation of D-glucose concentration over the fermentation time in samples fermented at 0.1, 5, 100 MPa and samples with pre-treatment of 100 MPa for 180 minutes.

The results in Figure 26 show different D-glucose profiles accordingly to the conditions applied. For instance, when fermentation was carried out at atmospheric pressure, D-glucose concentration decreased over time (from 0.50 g/L at the beginning to 0.24 g/L after 600 minutes), indicating that this sugar is being consumed by the bacterial strains present in the sample. D-glucose is not very abundant in unprocessed milk. However, during fermentation, this compound is formed naturally due to lactose hydrolysis, which occurs intracellularly.

At 5 MPa, D-glucose concentration slightly increased (from 0.50 to 0.59 g/L) during the first 180 minutes, which may indicate that microbial cells are transporting and hydrolyzing extracellular lactose, forming D-glucose (and D-galactose), subsequently expelled to the extracellular medium. There is no report in literature of such a case like this, since usually the fermentative bacteria only expel D-galactose and use D-glucose in the glycolytic pathway [7]. However, given the harsh conditions to which the cells are exposed, it may be possible that some metabolic modifications are occurring. For example, β-galactosidase activity may be affected by HP, leading to a more extensive lactose hydrolysis, which will form D-glucose concentrations higher than the expected and inciting the cell to expel this excess.

Despite of the observed increase at the first 180 minutes, D-glucose concentration tended to decrease during the remaining fermentation time at 5 MPa,

reaching a final value of 0.33 g/L, which is significantly different ($p < 0.05$) from that estimated to samples always at atmospheric pressure.

In samples exposed to 100 MPa, it was observed a constant increase in D-glucose content over time, reaching a concentration of 1.52 g/L after 600 minutes at these conditions. These results may be discussed on the basis of the reasoning presented before to 5 MPa: the bacterial strains seem to be transporting lactose to inside the cell and performing lactose hydrolysis; however, since D-glucose is not being used in the glycolytic pathway (at 100 MPa, lactic acid fermentation is inhibited), D-glucose accumulated by the cell is expelled to the extracellular medium.

In the case of samples subjected to a pre-treatment of 100 MPa for 180 minutes (prior to the fermentation at atmospheric pressure), it was observed that D-glucose concentration increased from 0.50 to 0.67 g/L during the pre-treatment. Then, when the samples were transferred to atmospheric pressure, D-glucose concentration continued to increase, reaching a final value of 0.95 g/L. Accordingly to the previously discussed results, after the pre-treatment time, lactic acid fermentation is taking place and, in consequence, a substantial portion of sugars is used by the cell in the glycolytic pathway. It may be hypothesized that bacterial cells are consuming and hydrolyzing more sugars than necessary and subsequently, the excipient sugars are being released.

It is important to note that D-glucose corresponds to a reducing sugar and it is quantified by DNS method, used in this work to analyze the concentration of reducing sugars in each sample. Therefore, D-glucose formed and released (when the cells are exposed to HP) is certainly quantified by DNS method. However, D-glucose concentration values detected in this section are relatively low (maximum of 1.52 g/L) and, consequently, they hardly affect the total content of reducing sugars in the samples, which is much higher (ranges between ≈ 25 - 60 g/L).

In order to assess the feasibility of these results, the theoretical maximum amount of glucose in the samples was estimated, assuming that all lactose in milk would be hydrolyzed and none would be consumed (Appendix IV). It was concluded that the maximum amount of D-glucose detected in the samples by the analytical test kit (1.52 g/L after 600 minutes at 100 MPa) corresponded to approximately 5% of the estimated theoretical maximum. Therefore, D-glucose concentration values obtained in this section seem to be a minor fraction of the total glucose potentially in the samples. In fact, these values might not be very significant concerning the total sugar transportation

and metabolism in these cases. Nevertheless, the results clearly show differences when fermentation occurs under pressure or when a pressure pre-treatment is applied.

1.3.2. L- and D-Lactic Acid Concentration

Both lactic acid isomers were quantified and the obtained results are represented in Figures 27 and 28, for to L- and D-lactic acid concentration, respectively.

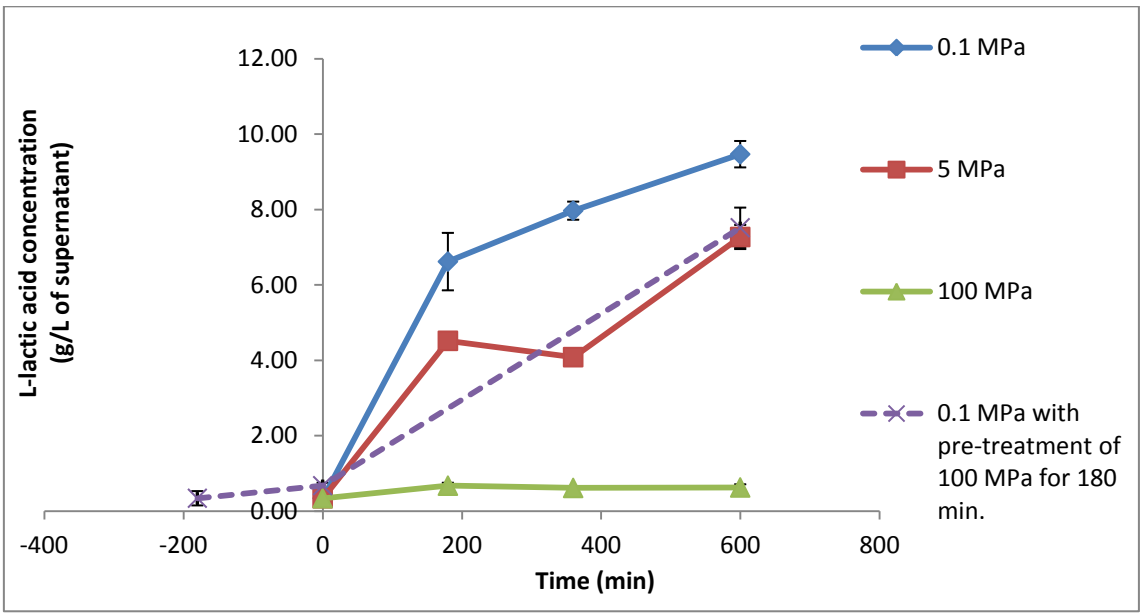

Figure 27. Variation of L-lactic acid concentration over the fermentation time in samples fermented at 0.1, 5, 100 MPa and samples with pre-treatment of 100 MPa for 180 minutes.

According to the observed in Figure 27, at atmospheric pressure, L-lactic acid concentration increased over time (from 0.34 to 9.47 g/L), with a higher rate during the first 180 minutes (which correspond to the typical fermentation time). This behavior indicates that the production of L-lactic acid by the bacterial strains is more accentuated in the first hours of fermentation. *S. thermophilus*, which produces the L-isomer, is sensitive to pH variation and, in consequence, is inhibited by the pH reduction during lactic acid fermentation [4]. This might explain the higher rate of L-lactic acid production during the first 180 minutes of fermentation.

When the fermentation process was performed under 5 MPa, the behavior was slightly different. As observed at 0.1 MPa, L-lactic acid concentration has increased over time (from 0.34 to 7.27 g/L) and the variation was more accentuated in the first 180 minutes. However, during the time period between 180 and 360 minutes, L-lactic

acid concentration stabilized (in which seems to be an experimental error) and, after that, the concentration increased once more, at a rate similar to the verified in the first minutes. It was also observed that, at these conditions, L-lactic acid concentration have not reached the values obtained at atmospheric pressure, which is consistent with the results discussed in previous sections, showing that acid production during fermentation at 5 MPa were always lower than at atmospheric pressure.

At 100 MPa, L-lactic concentration increased much less, but it is possible to note that, after 600 minutes, L-lactic acid concentration in the samples almost doubled, from 0.34 g/L, in the beginning, to 0.63 g/L.

Concerning HP pre-treated samples, it was observed that, during the pre-treatment time, L-lactic acid concentration practically doubled (from 0.34 to 0.68 g/L), but it still remained very low. After that, at atmospheric pressure, there was a marked increase of L-lactic acid concentration and, after 600 minutes, it reached a value of 7.52 g/L, which is significantly lower ($p < 0.05$) than the concentration at atmospheric pressure for the same time (9.47 g/L), but similar ($p > 0.05$) to the observed at 5 MPa (7.27 g/L). It is possible to conclude that *S. thermophilus* retained the viability during the pre-treatment at 100 MPa for 180 minutes and partially recovered its metabolic activity when subsequently exposed to “optimal” growing conditions. These results are in accordance with those previously discussed to titratable acidity of samples subjected to this pre-treatment.

As previously indicated, the results of D-lactic acid concentration in samples at different pressure conditions are represented in Figure 28.

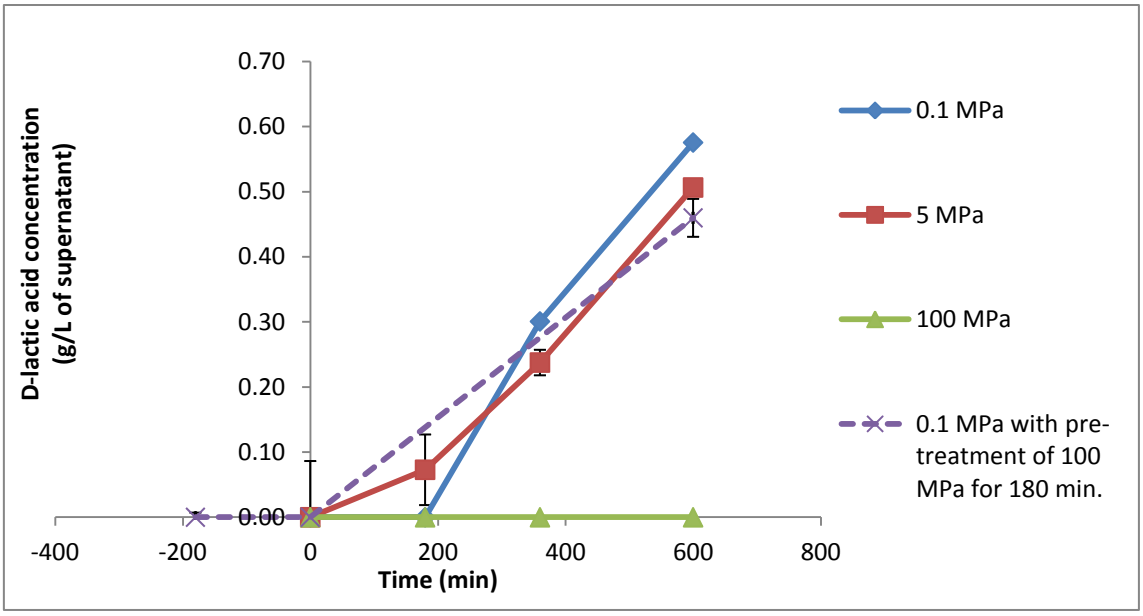

Figure 28. Variation of D-lactic acid concentration over the fermentation time in samples fermented at 0.1, 5, 100 MPa and samples with pre-treatment of 100 MPa for 180 minutes. The values presented as 0.000 g/L correspond to D-lactic acid concentration values below the quantification limit.

The values represented as 0.000 g/L in Figure 28 correspond to samples with D-lactic acid concentration below the quantification limit of the analytical test kit. It is possible to observe that the values obtained for D-lactic acid concentration are quite lower than those estimated to the L-isomer. As previously stated, during yogurt fermentation both lactic acid isomers are simultaneously produced (*S. thermophilus* produces L-lactic acid, while *L. bulgaricus* produces D-lactic acid). However, D-lactic acid may cause metabolic disorders when ingested in excess, thus the industrial starters used in yogurt production have low proportion of *L. bulgaricus* and, in consequence, a low proportion of D-lactic acid, as verified in this work [5, 7].

When fermentation was carried out at atmospheric pressure, D-lactic acid concentration was not quantifiable in the first 180 minutes, due to the low concentrations of analyte accumulated during this period. However, at 360 minutes of fermentation it was observed a D-lactic acid concentration of 0.30 g/L and, at the end of the process, this value increased to 0.58 g/L.

At 5 MPa, D-lactic acid concentration was quantifiable after 180 minutes of fermentation and it has increased over time, reaching a final value of 0.51 g/L, which is similar ($p > 0.05$) to the observed at atmospheric pressure. It seems that *L. bulgaricus*

metabolic activity is not much affected by these pressure conditions, at least in what concerns to D-lactic acid production.

Regarding the samples at 100 MPa, it was not possible to quantify D-lactic acid concentration at any time, since all values were bellow the quantification limit. These results indicate that, at 100 MPa, the production of this isomer was not detected, probably due to *L. bulgaricus* inhibition or destruction.

In the case of pre-treated samples, during the pre-treatment at 100 MPa for 80 minutes, D-lactic acid concentration values were below the quantification limit. However, during the subsequent fermentation at atmospheric pressure, D-lactic acid concentration increased over time, reaching a final value of 0.46 g/L, which is significantly lower ($p < 0.05$) than the observed at atmospheric pressure, but similar to the result at 5 MPa ($p > 0.05$). It seems that the pre-treatment of 100 MPa for 180 minutes did not destroyed *L. bulgaricus*, which was probably inhibited during the time under HP and partially recovered its metabolic activity when subsequently at 0.1 MPa, showing that this bacterial strain was able to overcome this stress shock.

The ratios between L- and D-lactic acid concentrations are calculated in Appendix V. It was not possible to obtain ratio values to all samples, since D-lactic acid was not detected in some cases. The obtained results show that, after 180 minutes at 5 MPa, the L-:D- lactic acid proportion was 61.9, which means that, at these pressure conditions, the concentration of L-lactic acid was ≈ 60 fold higher than the D-isomer. It was not possible to compare this value with the correspondent ratio at atmospheric pressure, since D-lactic acid was not quantified after 180 minutes at 0.1 MPa. Interestingly, it was verified that D-lactic acid was produced earlier at 5 MPa than at atmospheric pressure. At the end of fermentation (600 minutes), the L-:D- lactic acid ratios were similar at atmospheric pressure, 5 MPa and in pre-treated samples. These results are important, since it shows that despite of the differences during the fermentative process, the final product has the same proportion of both lactic acid isomers. As outlined above, this parameter is particularly relevant due to the health problems that may emerge from the excessive consumption of D-lactic acid.

In conclusion, it was observed that all yogurt samples (produced at different pressure conditions) had a higher concentration of L-lactic acid, relatively to the D-isomer. These results are consistent with the information present in literature, which refers that yogurt starters have a higher proportion of *S. thermophilus* than *L. bulgaricus* and, in consequence, yogurt is richer in L-lactic acid than in D-isomer [7]. Moreover, it

is known that *S. thermophilus* is initially more active than *L. bulgaricus* in relation to acid production [1]. However, during a more advanced stage of fermentation, *S. thermophilus* is inhibited by the pH reduction [4]. The results observed to fermentation at atmospheric pressure are, in fact, consistent with the affirmations above: the production of L-lactic acid is more pronounced during the first 180 minutes, after which the production rate decreases; on the other hand, the production of D-lactic acid occurs mainly after 180 minutes of fermentation and it is practically absent before that time.

Regarding the effects of HP on L- and D-lactic acid concentration, it was concluded that, in general, pressure reduces the production of both isomers, which is in accordance with the titratable acidity results. However, after 600 minutes of fermentation, the L-:D- lactic acid ratios at atmospheric pressure, 5 MPa and in pre-treated samples were very similar, showing that besides the differences between those samples, the final proportion of both isomers was almost the same.

1.3.3. Acetaldehyde Concentration

Acetaldehyde is a product of LAB metabolism and corresponds to one of the main compounds responsible for yogurt characteristic flavor [1, 7]. Acetaldehyde concentration was assessed in this work and the obtained results are represented in Figure 29.

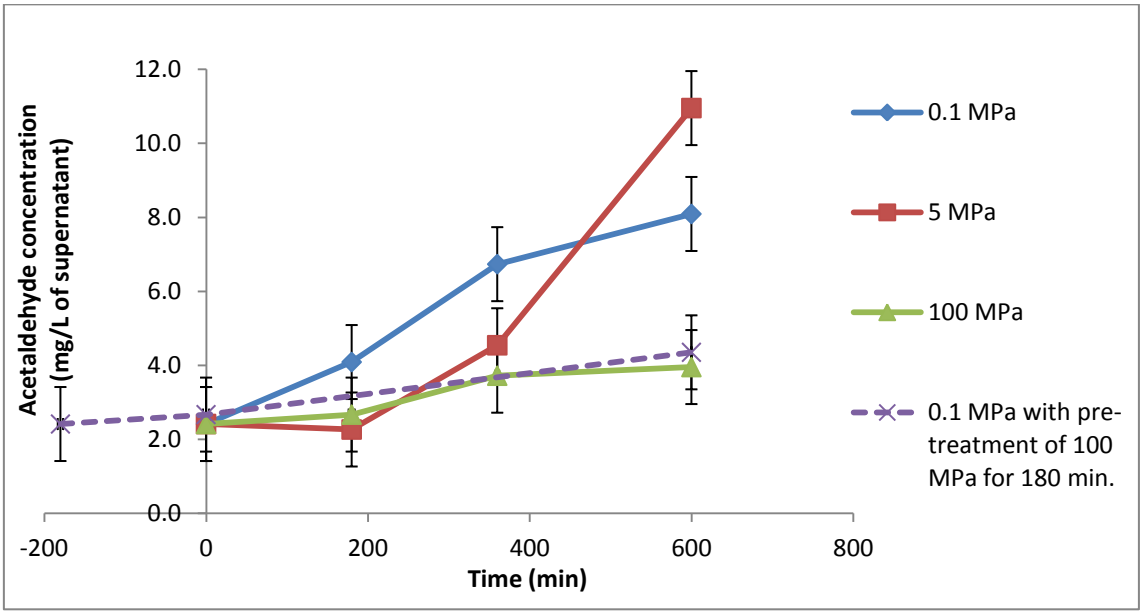

Figure 29. Variation of acetaldehyde concentration over the fermentation time in samples fermented at 0.1, 5, 100 MPa and samples with pre-treatment of 100 MPa for 180 minutes.

Acetaldehyde concentration values detected in some samples were very low and, in consequence, difficult to quantify. In samples fermented at atmospheric pressure, acetaldehyde concentration increased over time, reaching a final value of 8.1 mg/L, which is in accordance with the acetaldehyde concentration values presented in literature (in the range of 2 - 42 mg/L, depending on several factors) [1].

At 5 MPa, acetaldehyde concentration remained constant during the first 180 minutes of fermentation (from 2.4 to 2.3 mg/L), possibly because the bacterial strains were still adapting to the pressure conditions. However, after that time, acetaldehyde concentration increased considerably, reaching a concentration of 11.0 mg/L at 600 minutes of fermentation. It is possible to conclude that acetaldehyde content is higher when fermentation was carried out at 5 MPa (comparatively to the process at atmospheric pressure), indicating that yogurt obtained at these pressure conditions would certainly have a more intense flavor. This result was confirmed during an informal sensorial analysis performed by six work colleagues, who stated that samples fermented at 5 MPa for 600 minutes shown "a more intense yogurt flavor".

In the case of samples exposed to 100 MPa, acetaldehyde concentration increased from 2.4 g/L to 4.0 g/L after 600 minutes of fermentation. It would be expected that acetaldehyde concentration would remain constant over time, since at these conditions the fermentative process seems to be inhibited, as previously discussed.

Concerning to pre-treated samples, it was not detected substantial acetaldehyde production during the pre-treatment time (it varied from 2.4 to 2.7 g/L). However, after 600 minutes at atmospheric pressure, the samples shown an acetaldehyde concentration of 4.4 g/L, which is similar ($p > 0.05$) to the value observed in samples at 100 MPa. Therefore, the obtained final product will certainly show relevant flavor differences (relatively to yogurt fermented at atmospheric pressure) and, in future work, it will be interesting to perform sensorial analysis to those samples, in order to confirm these conclusions.

1.3.4. Ethanol Concentration

Ethanol quantification corresponded to an attempt to detect a possible metabolic shift caused by HP. A previous work in literature [144] reported that *Clostridium thermocellum* suffered a metabolic shift under pressure conditions, causing the production of higher ethanol concentration. Therefore, it would be relevant to evaluate ethanol concentration in this work, since a similar behavior could be possibly observed

under pressure. Usually, the bacterial starters involved in yogurt fermentation show a homofermentative metabolism, but it is possible that, under stressful conditions (e.g. under HP) the microbial strains may acquire a heterofermentative metabolism, producing several new products, such as ethanol.

The results obtained for this parameter were below the quantification limit of the method and, in consequence, the values could not be considered. In future work, it will be interesting to perform HPLC to the samples, in order to verify if there is, in fact, ethanol (as well as other compounds) production under HP.

2. Effect of High Pressure on Microbial Counts of Probiotic Yogurt

In order to assess the effect of different HP conditions on the viability of the starter and probiotic strains in the samples, microbial counts of *Streptococcus thermophilus*, *Lactobacillus bulgaricus* and *Bifidobacterium lactis* were performed and the results are represented in Figures 30, 31 and 32, respectively. These results were subjected to a statistical analysis, in order to confirm if samples at different pressure conditions are significantly different. The results of this analysis are expressed in Appendix II – section d).

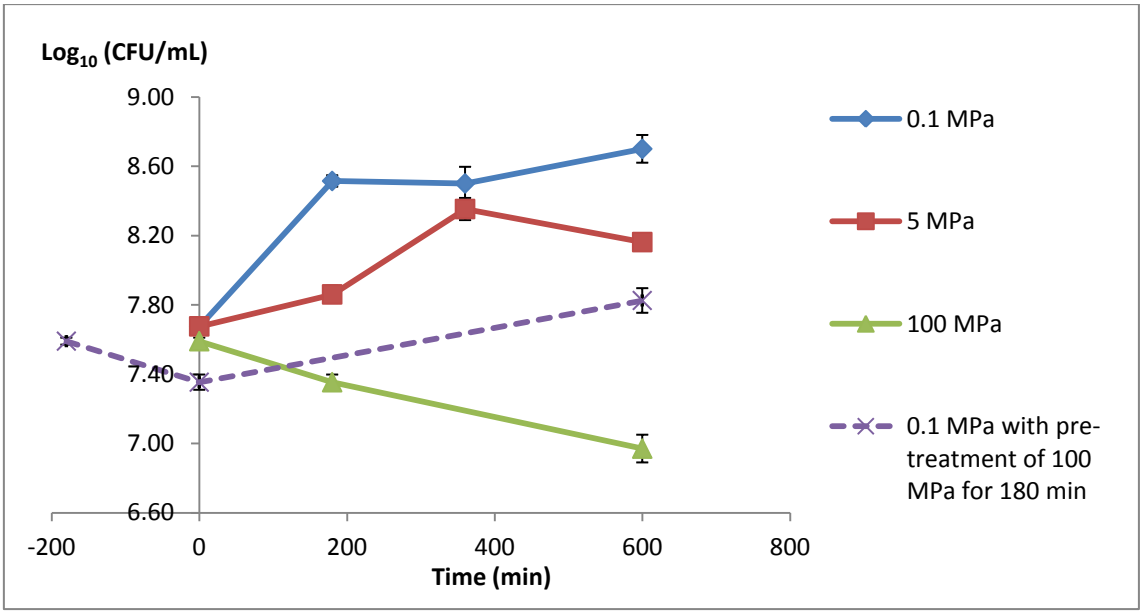

Figure 30. *Streptococcus thermophilus* count over the fermentation time in samples fermented at 0.1, 5, 100 MPa and samples with pre-treatment of 100 MPa for 180 minutes.

Analyzing Figure 30, it is possible to conclude that *S. thermophilus* load was already high in the initial samples (≈ 7.6 log CFU/mL). Concerning fermentation at atmospheric pressure, a marked increase in *S. thermophilus* load (from 7.68 to 8.51 log CFU/mL) was observed during the first 180 minutes. After this time, *S. thermophilus* load seemed to stabilize (8.70 log CFU/mL at 600 minutes of fermentation). These results are in accordance with literature, which refers that *S. thermophilus* growth occurs mainly during the first hours of fermentation, since it is inhibited by the pH reduction verified during lactic acid fermentation [4]. The discussed results are also consistent with those observed to L-lactic acid concentration (Figure 27), the main product of the primary metabolism of *S. thermophilus*. During the first stage of fermentation, *S. thermophilus* showed a higher growth rate and, in consequence, it was observed an accentuated production of L-lactic acid. Then, when the microbial culture was in stationary phase, L-lactic acid production tended to stabilize and it is possible that *S. thermophilus* acquired a secondary metabolism.

At 5 MPa, the microbial load of *S. thermophilus* was always lower than the observed to samples at atmospheric pressure, i.e. these pressure conditions were hampering *S. thermophilus* growth, despite of its ability to grow. During the first 180 minutes of fermentation, *S. thermophilus* count increased from 7.68 to 7.86 log CFU/mL and, after that, the growth rate was enhanced, indicating that, during the first fermentation hours, *S. thermophilus* culture was probably adapting to the pressure conditions. A slight culture decline during the time period between 360 and 600 minutes was also detected. At 600 minutes of fermentation, *S. thermophilus* load was 8.16 log CFU/mL, which is higher than the observed in the initial sample. It is important to highlight that *S. thermophilus* was able not only to survive but also to grow at 5 MPa.

The microbiological results at 5 MPa were not entirely consistent with those observed to L-lactic acid concentration at the same conditions (see Figure 27). L-lactic acid corresponds to a metabolic product and it would be expected that the production curve was similar to *S. thermophilus* growth. However, this behavior was not observed, since L-lactic acid concentration substantially increased from 360 minutes onwards (contrarily to what observed to cell growth during the same period), showing that, in this case, acid production is not directly related to cell growth. This effect is observed in several microorganisms, such as *S. cerevisiae* (Crabtree effect), which grows preferably at some conditions and ferments at different ones, proving that growth and fermentation are not always coupled [170].

These differences between microbial growth and L-lactic acid production may also be explained by the fact that, under HP conditions, microorganisms often suffer metabolic changes, possibly modifying the products formed during fermentation [146]. These metabolic changes are still not well understood, thus it is not possible to outline any further explanation to discuss the results of samples under HP.

In samples fermented at 100 MPa, it was verified that *S. thermophilus* load was reduced over time (from 7.59 to 6.97 log CFU/mL), indicating that these conditions are not suitable for *S. thermophilus* growth. The culture decline was reflected in the metabolic behavior of the strain and, as a result, there was no relevant production of L-lactic acid over time, as previously seen in Figure 27. However, it was observed that microbial reduction was not much accentuated (it was lower than a logarithmic reduction), which shows that *S. thermophilus* culture has a certain ability to withstand HP.

In the case of pre-treated samples, it was possible to note a decline of *S. thermophilus* culture during the pre-treatment time (from 7.59 to 7.35 log CFU/mL). After that, at atmospheric pressure, *S. thermophilus* load tended to increase, reaching a final value of 7.82 log CFU/mL, which is lower than the observed to samples at the same conditions without pre-treatment (8.70 log CFU/mL).

The results obtained for L-lactic acid concentration (see Figure 27) show that there was no considerable production of this compound during the pre-treatment, which is consistent with *S. thermophilus* decline over this time. Then, when the samples were transferred to atmospheric pressure, a marked increase in L-lactic acid concentration was observed, possibly as a consequence of *S. thermophilus* growth observed during this time. However, the increase of L-lactic acid concentration was more accentuated than the increase in *S. thermophilus* load, showing that these parameters are not always entirely related.

As previously indicated, the results of *L. bulgaricus* count are represented in Figure 31.

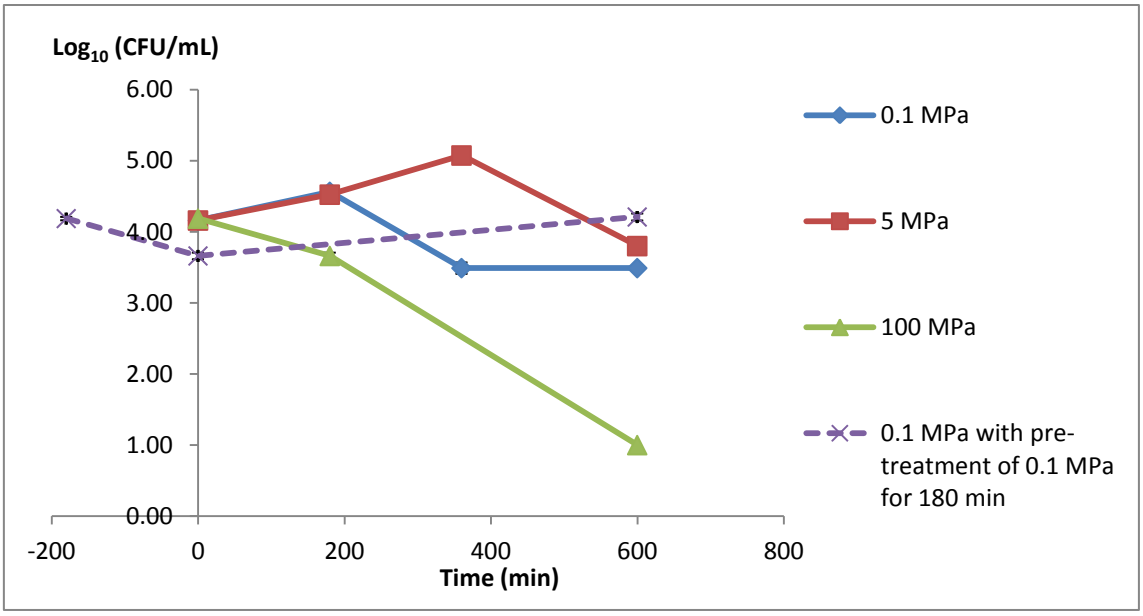

Figure 31. *Lactobacillus bulgaricus* count over the fermentation time in samples fermented at 0.1, 5, 100 MPa and samples with pre-treatment of 100 MPa for 180 minutes.

Samples fermented at atmospheric pressure shown an initial *L. bulgaricus* load of 4.16 log CFU/mL. During the first 180 minutes of fermentation, *L. bulgaricus* load was increased to ≈ 4.60 log CFU/mL and, after that, was decreased to 3.49 log CFU/mL, remaining nearly stable until the end of the process. These results indicate that, after the highest growth rate in the beginning of fermentation, *L. bulgaricus* load tends to decrease, probably due to the production and accumulation of undesirable compound(s), which will hurdle the growth of this microorganism.

According to the results of D-lactic acid concentration (Figure 28), a product of *L. bulgaricus* metabolism, it would be expected a higher growth rate at the final stage of fermentation (and practically no growth during the first 180 minutes). However, as concluded before to *S. themophilus* count, the production of lactic acid isomers and the microbial growth are not always interrelated, due to the previously discussed reasons.

It was also concluded that, in samples fermented at atmospheric pressure, *L. bulgaricus* load was always lower than the observed for *S. thermophilus*, just as described in literature [7]. In the initial sample, *S. thermophilus* load was ≈ 3.5 logarithmic units higher than *L. bulgaricus*. After 600 minutes of fermentation, *S. thermophilus* count increased approximately one logarithmic unit, while *L. bulgaricus* suffered a count reduction.

At 5 MPa, *L. bulgaricus* load increased during the first 360 minutes, reaching a value of 5.07 log CFU/mL, which is much higher than the values observed at atmospheric pressure. After that time, there was a marked decrease in microbial count, probably due to substrate depletion and/or product inhibition. It is possible to conclude that *L. bulgaricus* is able to survive and to grow under these pressure conditions, but it is more pressure sensitive than *S. thermophilus*. These results are in accordance to other described in literature for a pressure treatment of 400 MPa, which showed that *S. thermophilus* was more resistant to HP than *L. bulgaricus* [158]. Once more, it was observed that *L. bulgaricus* growth is not coordinated with the production of D-lactic acid, since the time period at which microbial load was declining (from 360 minutes to the end) corresponds to a phase with high D-lactic acid production rate (see Figure 28).

At 100 MPa, it was verified a gradual decline in *L. bulgaricus* count over time. After 600 minutes, the microbial load is represented as 1.00 log CFU/mL, since it was not possible to find any bacterial colony in the Petri dishes with this sample. Therefore, it is possible to conclude that this harsh treatment caused a substantial destruction of *L. bulgaricus* in yogurt samples. In this case, the D-lactic acid concentration results were directly related with *L. bulgaricus* count, since it was not observed any production of this isomer in these conditions (see Figure 28). Once more, it may be concluded that *L. bulgaricus* was more pressure sensitive than *S. thermophilus*, which is in accordance with the information available in literature [158].

In what concerns to pre-treated samples, *L. bulgaricus* load decreased (from 4.19 to 3.66 log CFU/mL) during the 180 minutes of pre-treatment. However, after 600 minutes at atmospheric pressure, the microbial count was increased to 4.21 log CFU/mL, indicating that *L. bulgaricus* had the ability to recover from the pressure pre-treatment. In this case, the final load of *L. bulgaricus* was higher than the observed to atmospheric pressure and 5 MPa, in which the samples were already in a decline stage after 600 minutes of fermentation.

The previously discussed results are in accordance with those observed to D-lactic acid concentration (Figure 28): despite of the fact that this isomer was not quantified during the pre-treatment, after that (at atmospheric pressure) D-lactic acid concentration has increased over time. The final concentration of this compound was lower than the observed at atmospheric pressure (without pre-treatment) and at 5 MPa. Therefore, it may be concluded that the higher load of *L. bulgaricus* in pre-treated samples is not reflected in D-lactic production.

In conclusion, the results obtained for starters quantification are, in general, consistent with those observed to sugar consumption and acid production. With the increasing pressure, there is microbial growth inhibition (and destruction, in some cases), causing a reduction in sugar consumption and acid production, i.e. a reduction in the fermentative metabolism. It was also observed that *S. thermophilus* is more pressure resistant than *L. bulgaricus*.

As previously indicated, the results of *B. lactis* count are represented in Figure 32. Until this moment, the results discussed in this work have not provided any direct information about the effect of HP on *B. lactis* viability and growth. On the one hand, the production of lactic acid and acetaldehyde by this probiotic strain is nearly insignificant. On the other hand, the sugar consumption over time is certainly affected by *B. lactis* metabolism, but these values reflect the substrate consumption by all three microbial strains in the inoculum and it is not possible to distinguish the effect of each one of them in the obtained reducing sugar concentration values. Therefore, the quantification of *B. lactis* was particularly important in this work, since it allows the understanding of pressure effects on its viability. These results do not provide information about the influence of HP conditions in the biological activity of *B. lactis*. It has not yet been possible to perform this study, due to temporal and material constrains, but it must be accomplished in future works, due to the high relevance of this subject.

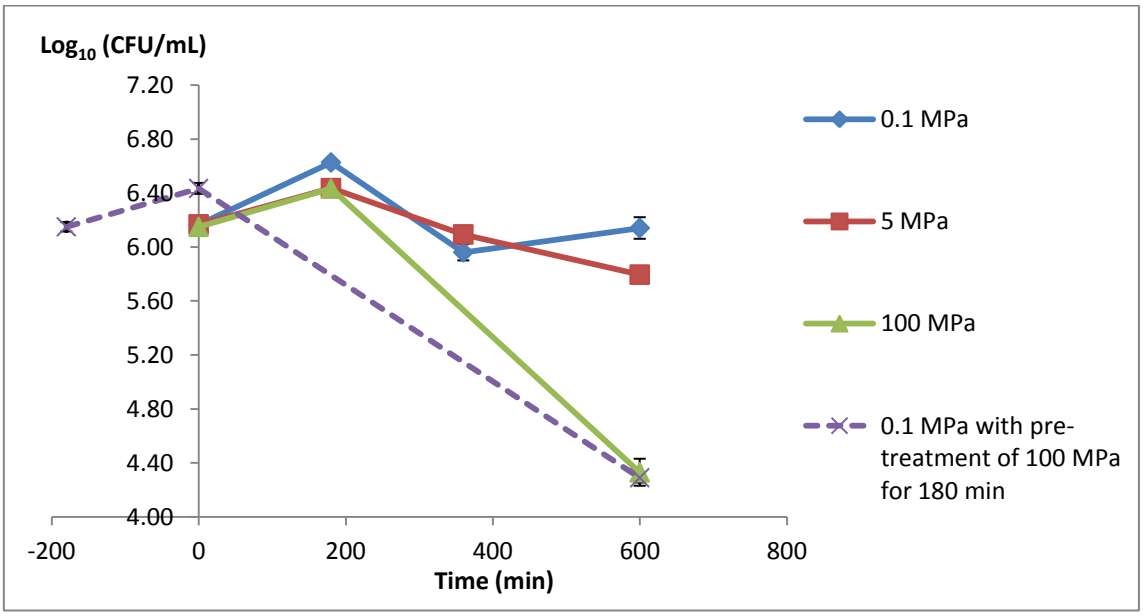

Figure 32. *Bifidobacterium lactis* count over the fermentation time in samples fermented at 0.1, 5, 100 MPa and samples with pre-treatment of 100 MPa for 180 minutes.

The microbial load of *B. lactis* in the initial sample was approximately 6.15 log CFU/mL, which is lower than the observed to *S. thermophilus* load, yet higher than the load of *L. bulgaricus* in the same sample.

When the fermentation was carried out at atmospheric pressure, it was observed an increase (from 6.17 to 6.63 log CFU/mL) in *B. lactis* load during the first 180 minutes, followed by a count decrease to 5.96 log CFU/mL, during the time period between 180 and 360 minutes. After that, *B. lactis* count slightly increased, reaching a final value of 6.14 log CFU/mL, which is not much different from the observed in the initial sample. In fact, *B. lactis* load in the sample seems to be nearly constant over the fermentation time.

In what concerns to fermentation at 5 MPa, *B. lactis* load increased during the first 180 minutes (reaching a maximum value of 6.44 log CFU/mL) and it was gradually reduced after that time. The sample collected after 600 minutes of fermentation shown a *B. lactis* load of 5.79 log CFU/mL, which is significantly lower ($p < 0.05$) than the observed to atmospheric pressure. However, it is important to highlight that this probiotic strain is capable of surviving and growing when exposed to 5 MPa (during 600 minutes), despite of the fact that these conditions are certainly not optimal for this microorganism.

Contrarily to what was expected, at 100 MPa, *B. lactis* load slightly increased during the first 180 minutes (from 6.15 to 6.43 log CFU/mL), possibly showing that this probiotic strain is slightly piezotolerant. In fact, this behavior was not detected in any other microorganism analyzed in this work, even thought *S. thermophilus* has shown some piezotolerant behavior as well. The values obtained after 360 and 600 minutes at 100 MPa indicate that *B. lactis* culture was declining (≈ 2 logarithmic reductions). At the end of the process, there were still viable bacteria, indicating that these pressure conditions did not cause a complete destruction of *B. lactis* in the sample.

In the pre-treated samples, there was a slight increase in *B. lactis* load during the pre-treatment time at 100 MPa, such as previously discussed. It was expected that after that, at atmospheric pressure, the growth would accelerate, since the conditions were more suitable for *B. lactis* growth. However, it was observed that, after 600 minutes at atmospheric pressure, the samples had a probiotic load similar ($p > 0.05$) to those exposed to 100 MPa during the same time period. Therefore, it might be concluded that despite of surviving (and even growing) at 100 MPa during the first 180 minutes, *B. lactis* cells probably suffer irreversible damage during this period. Yet, it is intriguing

how *B. lactis* count increases during the pre-treatment if the conditions are lethal and injuring. Ultimately it may be considered that the observed culture growth at the pre-treatment time was a result of an experimental error and, in future work, it will be important to repeat the analysis, to assess the integrity of these results.

As a conclusion, *B. lactis* was not destroyed and was capable of growing under 5 MPa. Moreover, the behavior was not much different from the observed at atmospheric pressure. In fact, in both cases, the variation in *B. lactis* load was not very accentuated over the fermentation time. On the other hand, when the samples were exposed to 100 MPa, the applied conditions have shown a great impact on *B. lactis* viability, but the microbial reduction did not exceed 2 logarithmic units, indicating that some viable cells were still in the sample after 600 minutes at 100 MPa. It was observed that the cultures pre-treated with 100 MPa for 180 minutes did not recover from the shock when subsequently exposed to atmospheric pressure. In this case, after 600 minutes at atmospheric pressure, the pre-treated samples have shown a culture decline of approximately 2 logarithmic reductions, similarly to what observed in samples at 100 MPa.

V. CONCLUSIONS

Currently, there are no published studies concerning the effect of HP on yogurt production. On this regard, the present work analyzed several physicochemical and microbiological parameters, in order to observe the effect of different pressure treatments in this lactic acid fermentation process.

Firstly, it was concluded that the fermentation monitoring parameters (pH, titratable acidity and reducing sugars concentration) were clearly affected by HP: with the increasing pressure, a lower variation in pH, titratable acidity and reducing sugars concentration was observed over time. At 5 MPa the fermentation rate was lower than at atmospheric pressure and, in consequence, it was necessary more time to reach the pH required to obtain yogurt. With the increasing pressure, it became harder to achieve the required pH/acidity and ultimately, at 100 MPa, the fermentation process ceased. These results show that HP causes the slowdown of lactic acid fermentation, probably due to the inhibition of metabolic activity of the starter strains involved in this process and, in some more extreme cases, the bacterial strains may even lose the capacity to survive at such harsh conditions.

Through the calculation of fermentation activation volumes, it was possible to confirm that the reactions involved in lactic acid fermentation are slowed down by pressure. In addition, it was concluded that the reducing sugars concentration corresponds to the parameter with the most sensitivity to pressure. In addition, it was verified that pH reduction is more affected by pressure than the acid increase, suggesting that acid production is not always reflected in the pH value. This effect may be explained by the production of different proportions of organic acids (with different pKa values).

Additionally, yogurt production was performed under combined pressure conditions, i.e. samples were subjected to a HP pre-treatment and the subsequent fermentation was carried out at atmospheric pressure. In general, there was no substantial variation in fermentation monitoring parameters during the pre-treatment time (to all tested conditions). After that, at atmospheric pressure, it was detected substrate consumption and acid production over time, showing that fermentation was taking place. It was possible to conclude that although the fermentative metabolism was inhibited during the pre-treatment, the starter strains were able to survive at these

conditions and later, at atmospheric pressure, its metabolic activity was partially re-acquired.

D-Glucose concentration was also monitored and a slight decrease over time was observed at atmospheric pressure and at 5 MPa, suggesting that the bacterial strains were consuming D-glucose to use in lactic acid fermentation. In contrast, in samples at 100 MPa and in pre-treated samples, it was observed a constant increase in D-glucose concentration over time. Possibly, the bacterial cells were transporting and hydrolyzing lactose, but since D-glucose is not being used in the glycolytic pathway (fermentation seems to be inhibited), this sugar might be expelled to the extracellular medium, leading to the increase of its concentration in the fermentative medium.

Lactic acid isomers were also quantified and the obtained results are in accordance with those observed to titratable acidity. In addition, it was concluded that, to all different tested pressure conditions, the yogurt samples had a higher concentration of L-lactic acid relatively to the D-isomer, which is consistent with the information in literature.

It was observed that acetaldehyde concentration tended to increase over the fermentation time, in all evaluated samples. The highest acetaldehyde content was detected in samples at 5 MPa (it was even higher than at atmospheric pressure) and, in consequence, the yogurt obtained at these pressure conditions may show a more intense flavor. In the case of samples at 100 MPa and pre-treated samples, acetaldehyde concentration was very low and probably these samples will have significant flavor discrepancies relatively to yogurt fermented at atmospheric pressure.

In what concerns to ethanol quantification, the estimated values were below the quantification limit of the method, suggesting that ethanol production is not observed during yogurt fermentation, even at stressful HP conditions.

A microbiological analysis was also performed, to evaluate the effects of HP on the viability of *S. thermophilus*, *L. bulgaricus* and *B. lactis*. The results have shown that the starter strains were able to survive and to grow under 5 MPa, contrarily to what observed at 100 MPa. In the case of pre-treated samples, the cells have shown ability to recover from the pressure shock and to re-acquire its metabolic activity. It was also concluded that *S. thermophilus* is more pressure resistant than *L. bulgaricus*, which is important since *S. thermophilus* seems to have a more relevant role in yogurt fermentation (it is present in higher proportion and produces a higher concentration of lactic acid).

The results of *B. lactis* count showed that this probiotic strain was not destroyed and it was capable to grow under 5 MPa. On the other hand, at 100 MPa it was observed a great impact on *B. lactis* viability. Cultures pre-treated with 100 MPa for 180 minutes have not recovered from the pressure shock when subsequently exposed to atmospheric pressure, possibly because cell damage caused in *B. lactis* cells by HP (100 MPa) was irreversible.

VI. FUTURE PROSPECTS

In what to concerns to future prospects, there are several paths that can be followed. First of all, it is important to accomplish the goals purposed in this study, which include the evaluation of the pressure effects on yogurt sensorial quality. Therefore, a sensorial analysis of the obtained samples, together with some specific analytical tools (such as rheology measurement and microstructure analysis), should be performed in the future.

Another interesting route corresponds to the analysis of yogurt biological activity, i.e. the potential health benefits promoted by the regular consumption of probiotic strains present in yogurt (which in this case include *B. lactis, L. bulgaricus* and *S. thermophilus*). The biological activity of these strains may be affected by the different pressure treatments applied in this work and it would be important to assess its behavior (*in vitro* and *in vivo*) at these conditions.

At last, it would be certainly challenging to evaluate not only the effects of pressure, but also the influence of temperature as a variable parameter in yogurt fermentation process. In these terms, it would be possible to establish the binomial pressure/temperature conditions which may lead to a faster and more efficient process.

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APPENDICES

I. DNS Reagent Preparation

II. Statistical Analysis

III. Activation Volumes Calculation

a. pH

b. Titratable Acidity

c. Reducing Sugars

IV. Estimation of Maximal Glucose Concentration in Yogurt

V. Determination of L-:D- Lactic Acid Ratios

Appendix I. DNS Reagent Preparation

10 g of DNS were weighted and dissolved in 200 mL of a 2N NaOH solution. The solution was then heated and stirred intensively. Simultaneously a solution of 300 g of potassium tartrate in 500 mL of distilled water was prepared and heated (with intense stirring). Both solutions were mixed and stirred. Distilled water was added to make up 1 L.

Appendix II. Statistical Analysis

A statistical analysis was performed in some sections of this work, allowing for a better comprehension and discussion of the obtained results. Significant differences ($p < 0.05$) between samples, for the same time of fermentation, are represented by different letters. Table cells filled with grey (without any value attributed) correspond to situations when the minimal required conditions to perform statistical analysis were not satisfied.

a) Monitoring of Fermentation under Different Pressure Conditions

Table 1. Statistical analysis performed pH values.

		Time (minutes)							
		0	**90**	**165**	**210**	**240**	**360**	**480**	**600**
Pressure (MPa)	**0.1**	*b*	*c*	*c*		*c*	*e*	*b*	*d*
	5	*b*	*bc*	*b*		*b*	*e*		*c*
	15	*b*	*b*	*a*		*a*	*d*		
	30	*b*	*a*		*c*		*c*	*a*	
	50	*a*	*a*		*b*		*b*		*b*
	100	*b*	*a*		*a*		*a*		*a*

Table 2. Statistical analysis performed to titratable acidity values.

		Time (minutes)							
		0	**90**	**165**	**210**	**240**	**360**	**480**	**600**
Pressure (MPa)	**0.1**	*a*	*a*	*a*		*a*	*a*	*a*	*a*
	5	*a*	*b*	*b*		*b*	*b*		*a*
	15	*a*	*b*	*b*		*b*	*c*		
	30	*a*	*bc*		*a*		*c*	*b*	
	50	*a*	*c*		*b*		*cd*		*b*
	100	*a*	*bc*		*c*		*d*		*c*

Table 3. Statistical analysis performed to reducing sugars values.

		Time (minutes)							
		0	**90**	**165**	**210**	**240**	**360**	**480**	**600**
Pressure (MPa)	**0.1**	*a*	*b*	*b*		*b*	*c*	*b*	*d*
	5	*a*	*b*	*a*		*a*	*c*		*c*
	15	*a*	*b*	*a*		*a*	*b*		
	30	*a*	*a*		*b*		*b*	*a*	
	50	*a*	*a*		*b*		*b*		*b*
	100	*a*	*a*		*a*		*a*		*a*

b) Fermentation under combined pressure conditions

i. Pre-treatment of 100 MPa for 90 minutes

Table 4. Statistical analysis performed to pH values.

		Time (minutes)				
		0	**90**	**210**	**360**	**600**
Pressure (MPa)	**0.1**	*a*	*c*		*b*	*c*
	100	*a*	*a*	*a*	*a*	*a*
	0.1 (pre-treated)	*a*	*b*	*b*		*b*

Table 5. Statistical analysis performed to titratable acidity values.

		Time (minutes)				
		0	**90**	**210**	**360**	**600**
Pressure (MPa)	**0.1**	*b*	*a*		*a*	*a*
	100	*a*	*b*	*b*	*b*	*c*
	0.1 (pre-treated)	*b*	*b*	*a*		*b*

Table 6. Statistical analysis performed reducing sugars values.

		Time (minutes)				
		0	**90**	**210**	**360**	**600**
Pressure (MPa)	**0.1**	*c*	*b*		*b*	*c*
	100	*a*	*a*	*a*	*a*	*a*
	0.1 (pre-treated)	*b*	*b*	*b*		*b*

ii. Pre-treatment of 100 MPa for 180 minutes

Table 7. Statistical analysis performed to pH values.

		Time (minutes)					
		0	**90**	**210**	**360**	**510**	**600**
Pressure (MPa)	**0.1**	*a*	*c*	*c*	*c*	*b*	*c*
	100	*a*	*a*	*a*	*a*		*a*
	0.1 (pre-treated)	*a*	*b*	*b*	*b*	*a*	*b*

Table 8. Statistical analysis performed to titratable acidity values.

		Time (minutes)					
		0	**90**	**210**	**360**	**510**	**600**
Pressure (MPa)	**0.1**	*b*	*a*	*a*	*a*	*a*	*a*
	100	*a*	*c*	*c*	*c*		*c*
	0.1 (pre-treated)	*b*	*b*	*b*	*b*	*b*	*b*

Table 9. Statistical analysis performed to reducing sugars values.

		Time (minutes)					
		0	**90**	**210**	**360**	**510**	**600**
Pressure (MPa)	**0.1**	*a*	*a*	*b*	*b*	*a*	*b*
	100	*c*	*a*	*a*	*a*		*a*
	0.1 (pre-treated)	*b*	*a*	*b*	*b*	*a*	*b*

c) Monitoring of Other Physicochemical Parameters under Different Pressure Conditions

i. D-Glucose Concentration

Table 10. Statistical analysis performed to D-glucose concentration values. (*Pre-treatment of 100 MPa for 180 minutes)

		Time (minutes)			
		0	**180**	**360**	**600**
Pressure (MPa)	**0.1**	*b*	*b*	*c*	*d*
	5	*b*	*a*	*b*	*c*
	100	*b*	*a*	*a*	*a*
	0.1 (pre-treated)*	*a*			*b*

ii. L- and D-Lactic Acid Concentration

Table 11. Statistical analysis performed to L-lactic acid concentration values. (*Pre-treatment of 100 MPa for 180 minutes)

		Time (minutes)			
		0	**180**	**360**	**600**
Pressure (MPa)	**0.1**	*a*	*a*	*a*	*a*
	5	*a*	*b*	*b*	*b*
	100	*a*	*c*	*c*	*c*
	0.1 (pre-treated)*	*a*			*b*

Table 12. Statistical analysis performed to D-lactic acid concentration values. (*Pre-treatment of 100 MPa for 180 minutes)

		Time (minutes)			
		0	**180**	**360**	**600**
Pressure (MPa)	**0.1**			*a*	*a*
	5			*a*	*ab*
	100				
	0.1 (pre-treated)*				*b*

iii. Acetaldehyde Concentration

Table 13. Statistical analysis performed to acetaldehyde concentration values. (*Pre-treatment of 100 MPa for 180 minutes)

		Time (minutes)			
		0	**180**	**360**	**600**
Pressure (MPa)	**0.1**	*a*	*a*	*a*	*b*
	5	*a*	*a*	*a*	*a*
	100	*a*	*a*	*a*	*c*
	0.1 (pre-treated)*	*a*			*c*

d) Effect of High Pressure on Microbial Counts of Probiotic Yogurt

Table 14. Statistical analysis performed to *S. thermophilus* count. (*Pre-treatment of 100 MPa for 180 minutes)

		Time (minutes)			
		0	**180**	**360**	**600**
Pressure (MPa)	**0.1**	*a*	*a*	*a*	*a*
	5	*a*	*b*	*a*	*b*
	100	*a*	*c*		*d*
	0.1 (pre-treated)*	*b*			*c*

Table 15. Statistical analysis performed to *L. bulgaricus* count. (*Pre-treatment of 100 MPa for 180 minutes)

		Time (minutes)			
		0	**180**	**360**	**600**
Pressure (MPa)	**0.1**	*a*	*a*	*b*	*c*
	5	*a*	*a*	*a*	*b*
	100	*a*	*b*		
	0.1 (pre-treated)*	*b*			*a*

Table 16. Statistical analysis performed to *B. lactic* count. (*Pre-treatment of 100 MPa for 180 minutes)

		Time (minutes)			
		0	**180**	**360**	**600**
Pressure (MPa)	**0.1**	*b*	*a*	*a*	*a*
	5	*b*	*b*	*a*	*b*
	100	*b*	*b*		*c*
	0.1 (pre-treated)*	*a*			*c*

Appendix III: Activation Volumes Calculation

a) H^+ Concentration

To perform the calculation of the activation volumes, several values of pH variation along fermentation time (at different pressure conditions) with linear behavior were selected. Using the pH values it was possible to calculate the concentration of H^+ and its respective napierian logarithm, represented in Figure 1.

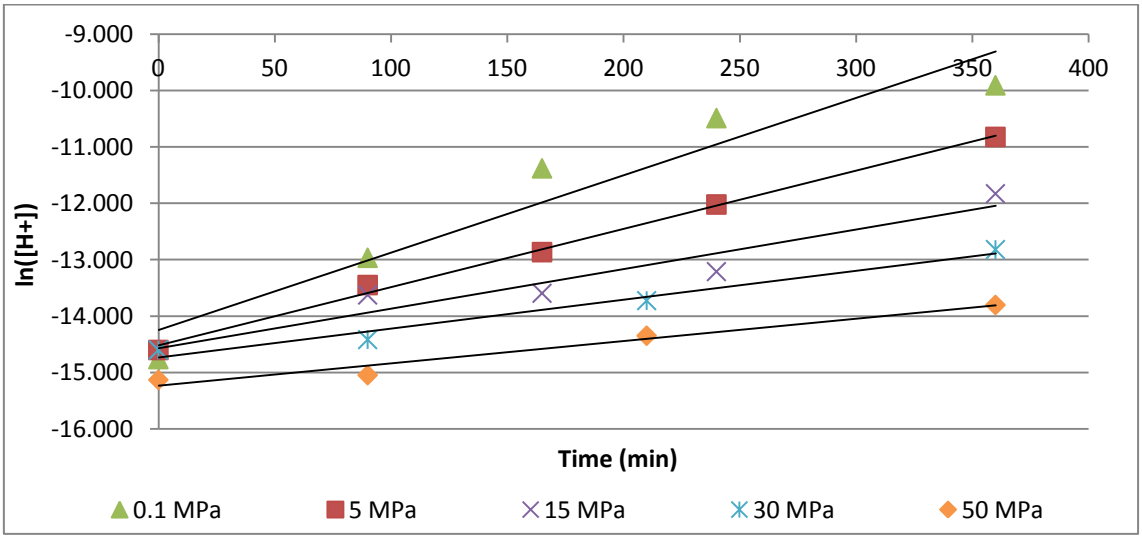

Figure 1. Napierian logarithm of H^+ concentration as a function of time, estimated to different pressure conditions.

Table 1 was constructed using the slopes of each series shown at Figure 1 as the reaction constant rate (k) and assuming that R_p = 8.314 (cm^3.MPa)/(K.mol) and T = 316.15 K.

Table 1. Determination of the reaction rate constant and respective napierian logarithm values over time, to different pressure conditions.

Pressure (MPa)	Pressure/(R_p*T)	ln([H^+]) vs. Time		
		|m| = k	r^2	ln(k)
5	1.9E-03	1.03E-02	0.996	-4.573
15	5.7E-03	7.00E-03	0.930	-4.961
30	1.1E-02	5.11E-03	0.976	-5.276
50	1.9E-02	3.95E-03	0.964	-5.534

The values shown in Table 1 were then used to calculate the linear relation present in Figure 2, which slope corresponds to the activation volume value obtained to pH (54.09 cm^3/mol).

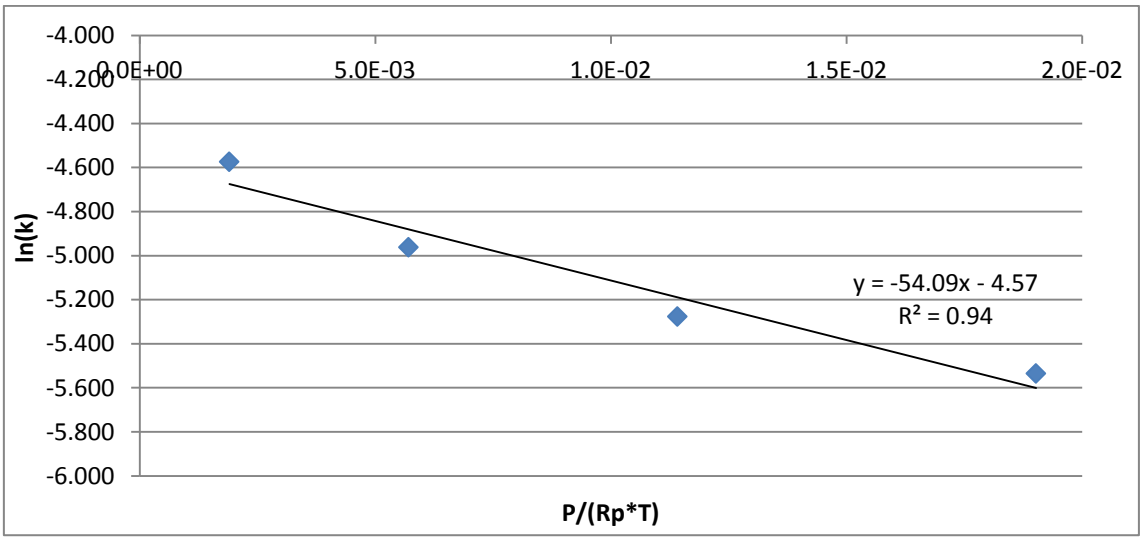

Figure 2. Activation volume calculation (correspondent to the slope of the linear equation).

b) Titratable Acidity

To perform activation volumes calculation, several values of titratable acidity variation along fermentation time (at different pressure conditions) with linear behavior were selected and its napierian logarithm was calculated (Figure 3).

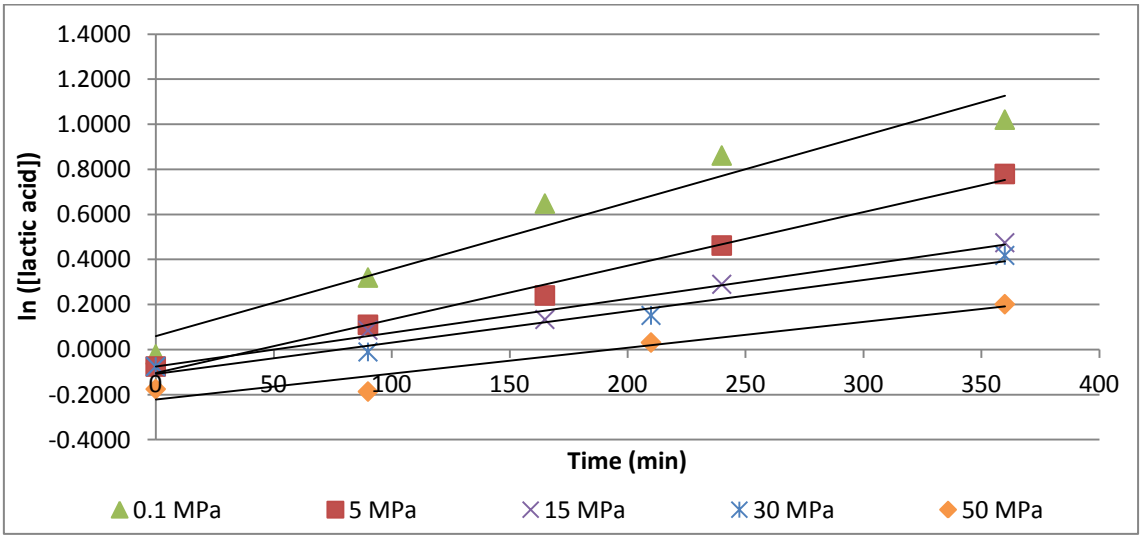

Figure 3. Napierian logarithm of lactic acid concentration as a function of time, estimated to different pressure conditions.

Table 2 was constructed using the slopes of each series shown at Figure 3 as the reaction constant rate (k) and assuming that R_p = 8.314 (cm^3.MPa)/(K.mol) and T = 316.15 K.

Table 2. Determination of the reaction rate constant and respective napierian logarithm values over time, to different pressure conditions.

Pressure (MPa)	Pressure/(R_p*T)	ln(Titratable Acidity) vs. Time		
		|m| = k	r^2	ln(k)
5	1.9E-03	0.00238	0.991	-6.040
15	5.7E-03	0.00150	0.987	-6.500
30	1.1E-02	0.00139	0.976	-6.577
50	1.9E-02	0.00115	0.932	-6.771

The values shown in Table 2 were then used to calculate the linear relation present in Figure 4, which slope corresponds to the activation volume value obtained to titratable acidity (37.36 cm^3/mol).

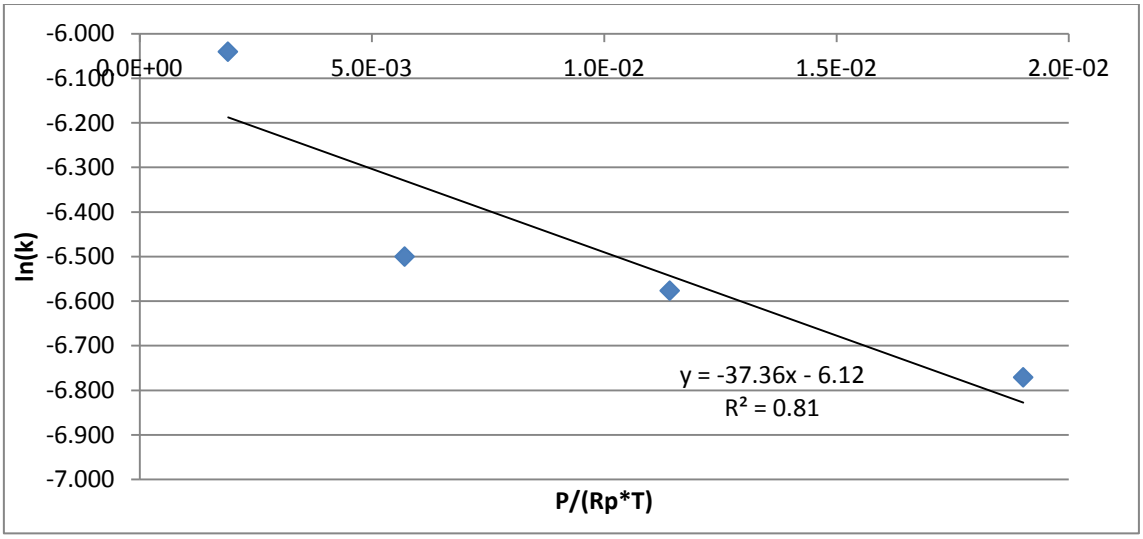

Figure 4. Activation volume calculation (correspondent to the slope of the linear equation).

c) Reducing Sugars Concentration

To perform activation volumes calculation, several values of reducing sugars concentration variation along fermentation time (at different pressure conditions) with linear behavior were selected and its napierian logarithm was calculated (Figure 5).

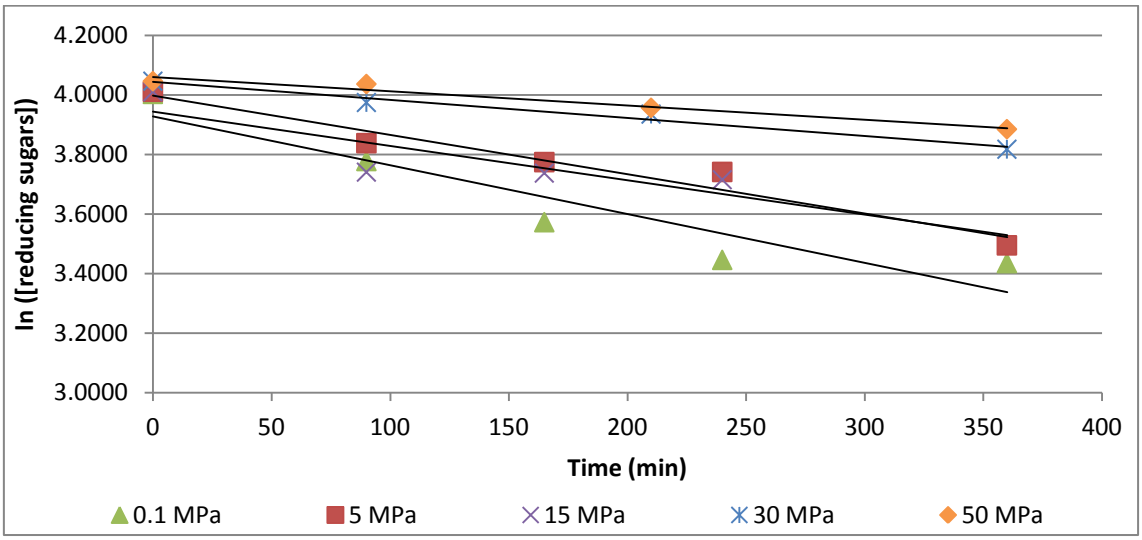

Figure 5. Napierian logarithm of reducing sugars concentration as a function of time, estimated to different pressure conditions.

Table 3 was constructed using the slopes of each series shown at Figure 5 as the reaction constant rate (k) and assuming that R_p = 8.314 (cm^3.MPa)/(K.mol) and T = 316.15 K.

Table 3. Determination of the reaction rate constant and respective napierian logarithm values over time, to different pressure conditions.

Pressure (MPa)	Pressure/(R_p*T)	ln(Reducing Sugars) vs. Time		
		|m| = k	r^2	ln(k)
5	1.9E-03	0.00132	0.955	-6.631
15	5.7E-03	0.00115	0.713	-6.766
30	1.1E-02	0.00061	0.976	-7.409
50	1.9E-02	0.00048	0.965	-7.650

The values shown in Table 3 were then used to calculate the linear relation present in Figure 6, which slope corresponds to the activation volume value obtained to reducing sugars concentration (64.01 cm^3/mol).

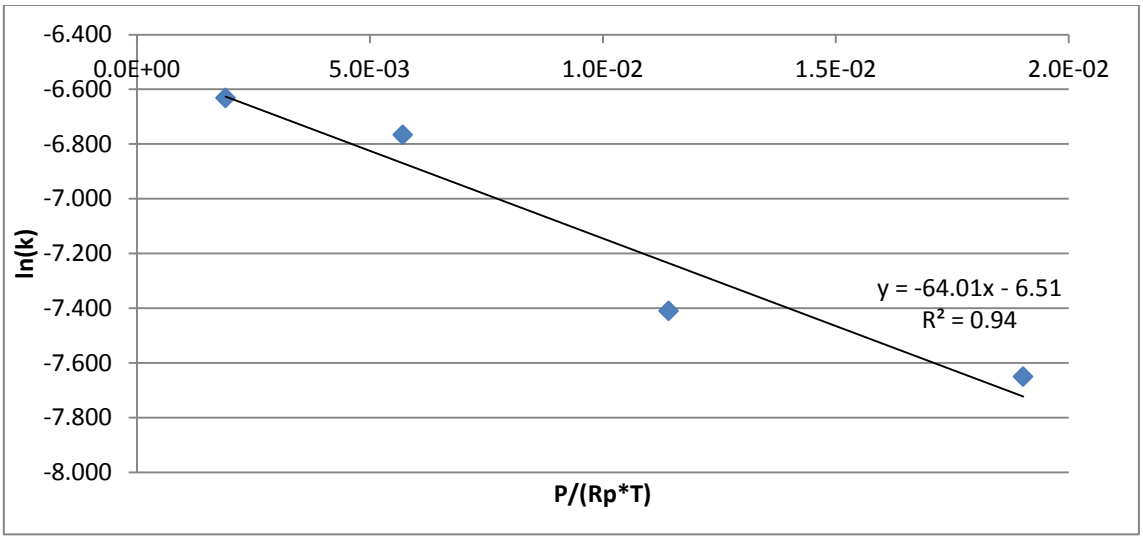

Figure 6. Activation volume calculation (correspondent to the slope of the linear equation).

Appendix IV. Estimation of Maximal Glucose Concentration in Yogurt

According to literature [4], the percentage of lactose in milk is ≈ 5% (in weight) and semi-skimmed milk density at 20 ^{o}C is 1.020 Kg/L. So, there are 5 mg of lactose in 0.098 mL of milk.

In the beginning of fermentation, we have 7.5 mL of sample with 0.38 g (1.1×10^{-3} mol) of lactose to be consumed by lactic acid bacteria. Assuming that lactose present in milk is totally hydrolyzed by the reaction represented in Figure 1 and no glucose is consumed, at the end of fermentation the samples would have 0.20 g (1.1×10^{-3} mol) of glucose (1 mol lactose:1 mol glucose).

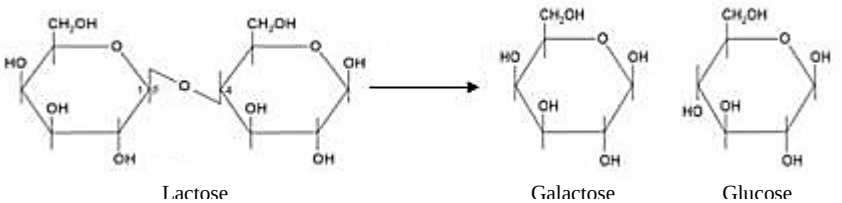

Figure 1. Schematic representation of lactose hydrolysis.

This estimated theoretical value was compared with the value obtained to the sample with maximal D-glucose concentration (1.52 g/L of supernatant). In this case, the collected supernatant has ≈ 0.011 g of D-Glucose, which corresponds to ≈ 5% of the estimated theoretical value, i.e. the D-glucose concentration values obtained in this work correspond to a small percentage of the total concentration which may be potentially present in the samples.

Appendix V. Determination of L-:D- Lactic Acid Ratios

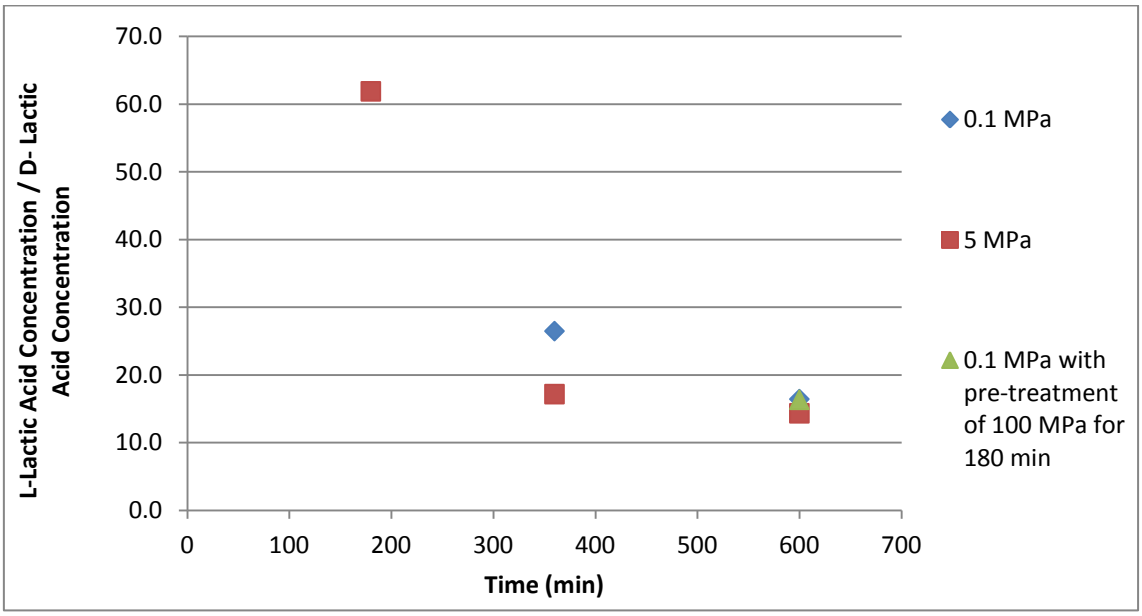

Figure 1. Ratios of L-:D- lactic acid concentrations during fermentation time.

On samples for which D-lactic acid was not detected the ratio value is not represented in Figure 1, since it is impossible to calculate the proportions of both lactic acid isomers.