Integrated Master in Bioengineering

Specialization of Biological Engineering

Optimization of lactic acid fermentation in the production of red pepper paste

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Dissertation for Master degree in Bioengineering

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ABSTRACT

Lactic acid fermentation is one of the oldest and safest technologies to preserve vegetables and there are many different ways to do it. Every vegetable can be fermented however the most famous and economically relevant are cabbage, cucumbers and olives. Cabbage is particularly important as it is the raw material for the production of sauerkraut and kimchi, the two most famous fermented vegetables in the world. On the other hand, red pepper is one of the most common fermented vegetable in Portugal, which is used to produce red pepper paste, usually used for tempering.

The present work was developed in association with Mendes Gonçalves, a company that produces vinegars, sauces and condiments. The main goal was the achievement of an improved process for the production of red pepper paste through lactic acid fermentation with optimal procedural conditions. An additional objective was to make other vegetable formulations to ferment and to obtain new products.

Fermentations of red pepper were carried out taking into account different temperatures, salt contents, lactic acid bacteria (microflora, *Lactobacillus plantarum* and *Leuconostoc mesenteroids* mixed starter cultures and commercial starter cultures) and the use of backslopping of fermentations with and without mixed starter cultures.

The best temperature to perform red pepper fermentations was 29 °C since pH reduction and acid production were higher and faster compared to the fermentation at 18 °C. Concerning the role of salt concentration, a faster fermentation occurred with 0%. However, 2% was evaluated on the remaining fermentations as it was the lower value that favour the lactic acid fermentation.

A comparison with the microflora shown that the use of mixed starter cultures provided the faster fermentation when observing pH and acidity results. Commercial starter culture had the same behaviour of mixed cultures, it decreased pH until 3.5 and increased acidity until 1.5% in less time, nearly 72 h instead of 144 h. Backslopping was carried out with 1 and 10% of whey from previous fermentations using mixed starter cultures and microflora. The best result was obtained with the two backsloppings using microflora which produced a fermented red pepper in nearly 36 h. Finally, sensory analysis revealed that no differences were detected from the red pepper paste developed and the red pepper paste produced nowadays in Mendes Gonçalves.

Keywords: lactic acid fermentation, red pepper paste, lactic acid bacteria, backslopping

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INTRODUCTION

1. Background

Fermentation is one of the oldest ways to preserve food and fermented foods have been consumed worldwide for thousands of years (McNeil and Harvey 2008). Vegetable fermentation is one of the oldest and safest technologies that have been practiced over years in different ways, depending on the culture and the region (Katz 2012). The most famous products made through vegetable fermentation are sauerkraut and kimchi and they come mainly from Germany and Korea, respectively.

Although fermented vegetables are not very representative in Portugal, pepper is one of the vegetables that is often used fermented. Red pepper paste used for tempering the food is probably the most famous fermented vegetable that is consumed in this country.

The present work was developed in association with Mendes Gonçalves, a Portuguese company located in Golegã, Santarém, which produces vinegars, sauces and condiments. Its history began in 1982 with the fig vinegar and, since then, this company always wanted to valorise Golegã, building there one of the most modern factories in Europe and one of the most famous between big distribution brands (Mendes Gonçalves 2016).

Mendes Gonçalves is the owner of Paladin, Creative and Peninsular and it intends to make them a reference anywhere in the world. For that purpose, despite its growth, it stills continue to maintain a close relationship with its customers, partners and employees and its centre of interest is innovation, where the company continues to invest internally.

Nowadays, Mendes Gonçalves is particularly recognized by Paladin, the brand that this company bought in 2000 and relaunched in 2013. The internationalization of Paladin has privileged the Maghreb and the Middle East and now is entering on the African countries in order to produce sauces and condiments based on the Angolan traditional flavours (Aicep Portugal Global 2016). Recently, the SIAL Innovation Paris 2016 rewarded Paladin due to the efforts that have been done in the field of innovation (Marketeer 2016). This focus allowed Mendes Gonçalves to win the relevance and prominence in the market and it was due to the many awards that their products received (Trends & Innovations, SIAL D'OR, Innoval, Gulfood Dubai) that this company captivated the interest of multinational companies.

Although all the interests on the production of innovative products, this company has also interest on the improvement of the older ones in order to achieve new and improved production conditions.

2. Research Objectives

The objective of this work was the study of lactic acid fermentations of red pepper. Initially, the main goal was the achievement of a controlled process for the production of red pepper paste with optimal procedural conditions and, after this achievement, the objective was to make other vegetable formulations to ferment and to obtain new products.

To achieve these objectives, this project aimed the determination and the analysis of physical, chemical and microbiological parameters before, during and after diverse lactic acid fermentations of red pepper.

Fermentations were carried out taking into account different temperatures, salt contents and microorganisms (the use of microflora, *Lactobacillus plantarum* and *Leuconostoc mesenteroids* mixed starter cultures and commercial starter cultures) and the use of backslopping of fermentations with and without mixed starter cultures.

3. Thesis organization

This work is divided in five chapters. Initially, the introduction section describes the background for this project and its research objectives.

Literature review describes how fermentation has influenced the history of conserved foods, particularly how vegetable fermentation has influenced the vegetable consumption over hears and its benefits. The principal fermentation influencing factors are described such as salt, the use of starter cultures and the anaerobic conditions. An overview in biochemistry of lactic acid fermentations was also made as well as a description of microorganisms involved in this kind of fermentation and its fermentative mechanisms. Finally, the principal types of fermented vegetables are described including red pepper paste.

Material and methods is the chapter where all the methodologies are described in order to accomplish the objectives of the present work. The types of fermentation carried out and the analysis for its evaluation are described in this section.

Fourth chapter presents the results and their discussion. In the fifth chapter the achievements of the present work are summarized and future work is suggested.

LITERATURE REVIEW

1. Fermentation – an ancient tradition

Fermentation is one of the oldest ways of food preservation technologies in the world. Traditional fermented foods such as bread, cheese and wine, have been consumed for thousands of years and are strongly linked to culture and traditions of many people, especially in rural households and village communities. The origins of fermentation are lost in ancient history but it is known that the Egyptians had knowledge on the techniques to convert starchy grains into alcohol (McNeil and Harvey 2008).

In fact, fermentation has been practiced by humankind for a long time, long before underlying scientific principles were understood. Scientific understanding of fermentation only began in the 1850s, after Pasteur isolate two different forms of amyl alcohol which were different in terms of activity, one of them was optically active (L-enantiomer) and the other was not (D-enantiomer) (El-Mansi et al. 2012). This observation led Pasteur to study the underlying reasons behind it and consequently to study fermentation. In 1857, fermentation was associated with life and with the structural integrity of the yeast cells as being living organisms that depend of fermentation to grow, reproduce and survive. This fact was concluded by Pasteur, which published his studies and marked the birth of the studies on the microbiology inside fermentation (El-Mansi et al. 2012).

Although fermentation has been used for many years, more research is needed for a better understanding of microbial and enzymatic processes responsible for the transformations of food, beverages, and so on. Since the twentieth century, fermentation has been applied to make a range of higher value products than simple ethanol such as therapeutic proteins and antibodies that are more complex and costly than the previous products. Basically, the quality of fermentation products that are made nowadays is determined by the fermentation stage so it is needed a clear understanding of what happens there, how it can be monitored, controlled and carried out (McNeil and Harvey 2008). Adapting this knowledge of fermentation control, it is possible to deal with the potential of using microorganisms towards meeting the growing world demand for food. In fact, fermentation plays a very important role in food preservation and nutritional enrichment that is an advantage in a world increasingly competitive and selective (Battcock and Azam-Ali 1998).

2. Fermented vegetables

According to the US Department of Agriculture, vegetable fermentation is one of the oldest and safest technologies to preserve vegetables. In fact, this kind of fermentation has been practiced for a long time, in many regions of the planet and in different ways. Some traditions wilt vegetables, others bruise them. Some people ferment just one kind of vegetables, others mix different vegetables together and put spices, fruit and other additives. Fermenting time can be days, months or even years. Most traditions work with native bacteria of vegetables and others add various starters. Concluding, there is no single way of accomplishing this task that has been so widely understood by different cultural traditions and incorporated into secret family recipes that passed through generations with adjustments and adaptations (Katz 2012).

It was on early 1900 that reports on the microbiology and biochemistry of vegetable fermentation started to appear in scientific literature. Early research on microorganisms present in fermented vegetables was done by Fred at the University of Wisconsin and after by Pederson, that studied sauerkraut fermentation at Cornell University from the 1930s to 1970s. After this period more studies were done about vegetables, investigations of the yeasts responsible for spoilage cucumber products and so on (McFeeters et al. 2013).

Nowadays, fermented vegetables are considered an artisanal food, a combination of traditional methods and scientific knowledge used to preserve flavours, colors and nutritive value (Shockey and Shockey 2014). Fermented vegetables started to be studied due to the increasing interest in several European countries. Just in Europe there are over twenty different commercial fermented vegetables, however, the most famous and economically relevant are olives, cucumbers and cabbage. The major part of olives and cucumbers are produced in Spain and nearly 50% of cabbages are fermented in Germany (Abriouel et al. 2008).

Despite the wide diversity in types of fermented vegetables produced around the world, the principles involved in the manufacture are very near the same.

3. General production

Vegetable fermentation has always some steps that are common in every type of fermentation (Katz 2012): chop or grate vegetables; add salt or a brine solution; add or

not starter cultures; pack the vegetables into a jar in order to put them submerged into the liquid; wait for the perfect flavour.

Therefore, there are many ways to produce this kind of vegetables. For a better understanding, every step will be explained below with further detail.

3.1. Chopped and grated vegetables

This particular step can be done in many different ways. Depending on the final purpose of the product; vegetables can be fermented in whole pieces or chopped which is easier not only to manage and treat, but also to ferment. This step is important because it increases the surface area, breaking down the cell structure, which facilitates pulling juice out of vegetables (Katz 2012; Shockey and Shockey 2014). Normally, grated vegetables are used with their own juices and water, if needed, and whole pieces of vegetables are used with brine solutions, to facilitate the fermentation.

3.2. Salt and brine solution

Vegetables can be fermented with or without salt. Salt is important due to the taste and the texture that it gives to the fermented vegetables. Besides that, it has the potential to ferment longer and slowly (Katz 2012) and facilitates the exit of water from vegetables through osmosis, which is important to submerge the vegetables under their own juices. Vegetable pectins are also influenced by salt since it hardens these proteins, preventing the action of pectin digesting enzymes in vegetables that could make them mushy, keeping vegetables crispier (Katz 2012; Shockey and Shockey 2014). Salt also creates a selective environment, narrowing the concentration range in which bacteria can grow, so it helps the salt-tolerant bacteria, the ones needed, having a competitive advantage. Furthermore, salt inhibits yeasts, which break down the sugars into alcohol instead of lactic acid, the product that it is needed (Shockey and Shockey 2014). Concluding, salt is extremely important since it extends the potential for preservation by slowing fermentation, slowing the pectin digesting enzymes activity and slowing the development of undesirable microorganisms (Katz 2012; Shockey and Shockey 2014).

There are two ways in which salt can be introduced into vegetables: dry-salting and brining. Dry-salting is simply sprinkling salt on vegetables and brining requires mixing salt with water. For the first method, it is necessary to have vegetables chopped or shredded since they have much more surface area exposed, which facilitates the exit

of water from vegetables through osmosis. On the other hand, when vegetables are left in large pieces, or even whole, brine method is more appropriate since vegetables interact with the brine in the process of osmosis too, dehydrating the vegetables cells, in which water is replaced by salt water (Shockey and Shockey 2014). This process facilitates the fermentation and the submersion of vegetables.

The amount of salt to be used is also a determining factor in fermentation. Normally, 0.8 % of salt to vegetable weight would prevent the decomposition talked above, however, the standard amount of salt is kept a little higher, around 2-3 % by weight for dry-salt method. This quantity also prevents the softer textures, most of times undesirable (Katz 2012; Shockey and Shockey 2014). For the brine method, the normal amount of salt is around 5 % weight of water. This value represents the salinity level, which is weight of salt (in grams) per volume (in mL) of what it is being dissolved into (Katz 2012). Nevertheless, the amount of salt is not very accurate since it depends, most of the times, of the tastes that people want to have in vegetables, always ensuring the quality and security levels. It is also very important to understand that, too low sodium content does not provide the concentration range in which the desirable bacteria can grow, but too much salt will inhibit fermentation by desirable bacteria (Shockey and Shockey 2014).

Another aspect of salting vegetables for fermentation is the type of salt that should be used. There are many types of salts but the one that is used the most, the common “table salt” is highly refined; it is the product of a chemical and high-temperature industrial process that removes all the valuable magnesium salts as well as naturally minerals present in the sea. The reason for studying which type of salt is the best for fermentation is that it influences the microorganisms involved in the fermentation. Iodine that is added to the refined salt, in order to help countries with iodine deficiency (Amr and Jabay 2004), do not have significant effect on the rate at which the salt penetrates through vegetables, on its taste and on vegetables contents of vitamin, however, it can significantly affect the colour and the texture, which becomes softer (Amr and Jabay 2004). This substance has antimicrobial properties which can cause darkening and cloudiness of fermented vegetables (Katz 2012). The alternatives are the use of pickling salts, or kosher salts. Pickling salt is a fine-grain salt with a uniform structure that contains no iodine or anti-caking additives. It is pure salt generally used in pickling foods due to its fine grain that allows an easy dissolution in water. Kosher salt only differs in the grains,

which are coarse grains that allow a relatively slow dissolution. Unrefined sea salts can also be used, however, they have the same problem that kosher salts have, they are made up of coarse grains, which are not favourable to build a brine solution as described above (Shockey and Shockey 2014).

Fermenting without salt is also possible. Salt, as said before, is the principal ingredient in fermented vegetables that slows fermentation, inhibits undesirable bacteria and molds and slows the enzyme activity. Without it, fermentation is made in shorter periods of time and contaminations can happen much more often. In addition, the taste itself of the vegetables is not so appealing (Katz 2012; Shockey and Shockey 2014).

3.3. Starter cultures

Vegetable fermentation do not need starter cultures since vegetables have native bacteria adequate to initiate fermentation. They are able to produce successful and delicious ferments, however, most of the times people use starter cultures to control the speed of fermentation and the kind of microorganisms involved since these cultures, sometimes much more developed, are able to prevent the growth of undesirable bacteria (Katz 2012). Starter cultures are those kind of microorganisms that are used in the production of cultured products such as some fermented vegetables. The use of these cultures helps to standardize the fermentation by controlling the microbial flora, as said before. Although this method is a huge advantage, when industrial production is required, there are few cultures designed for vegetable fermentations thus, studies on the development of effective inoculants to rapidly initiate vegetable fermentation are desired (Gardner et al. 2001). This topic will be discussed in detail on section 9 on the microorganisms involved in lactic acid fermentation.

3.4. Pack vegetables into a vessel

When vegetables are ready to start the fermentation, they must be placed within a vessel. The equipment usually used in the fermentation is going to be described below, in section 10.

The most critical factor for success during vegetable fermentation is getting all the vegetables submerged into the liquid, otherwise molds and other oxygen dependent organisms, in presence of oxygen, will use vegetables as substrate to produce undesirable products. To prevent this problem, pressing the vegetables can be a solution in order to release more juice from them and cover themselves. On the other hand, there are special

weights to place upon the vegetables to force them staying submerged (Katz 2012). Actually there are specific equipments that help in these problems (section 10).

3.5. Wait until the end of fermentation

Fermentations can take different periods of time to reach the perfect texture and flavor. There is not a specific time, it will depend on how the vegetables are required as final product since, during fermentation, flavors and textures change and acidity increases. This type of process has to be always adapted to the requirements for the final product and to the environmental conditions (Katz 2012). Tasting and following the process are probably the key to have a good final product.

4. Fermenting environment

Once fermented vegetables are a live food, there are many conditions that can influence and determine the final product. Temperature, light, oxygen, pH and time are the principals factors that influence fermentation.

Normally, temperature must be between 13 ºC and 24 ºC because this range is favorable for the growth of the desired bacteria (Shockey and Shockey 2014). It is very important to have an adequate and stable temperature to encourage fermentation. Although high temperatures accelerate the production of acid and reduce the time of fermentation, microorganisms might not have time to develop properly and they might produce off-flavors and an underdeveloped acidity. Contrary, with too cold temperatures, especially on early stages of fermentation, desired bacteria cannot reproduce fast enough to develop the needed acidity to keep the fermentation medium free of contaminations. To maintain the temperature constant it is important to keep the fermentation in a dark place, out of direct sunlight, to prevent temperature fluctuations but also light damage (Shockey and Shockey 2014).

As it was discussed before, vegetables should be submerged into brine to create an oxygen-free environment and restrain the growth of undesired bacteria and molds. Sometimes, CO_2 production can help vegetables to go to the surface and, to prevent this situation, it is important to press down vegetables in order to release CO_2 bubbles, replace its space by brine and maintain oxygen far away. Surface contaminations may occur and they can be took out, leaving the safe ferment underneath, however, fermentation should

not be disturbed many times otherwise yeast spores and oxygen may interfere with the process and aerobic bacteria may spoil all the fermentation (Shockey and Shockey 2014).

Time is probably the factor that strongly influences fermentation. There is not a standard formula in which optimum probiotic content is achieved. Vegetable fermentation involves a progression of diverse bacterial species which have different peaks at different points during fermentation. These different cultures and environmental conditions over time are the responsible for all the changes that can occur and that will characterize the final product (Shockey and Shockey 2014).

Finally, another factor that influences fermentation is pH. During vegetable fermentation, the production of lactic acid, as it will be detailed below, will low the pH. As oxygen, pH is another factor that will affect the microorganisms in fermentation. Neutral values will be more welcoming for the growth of many other microorganisms without any interest. Vegetable fermentation bacteria are known to prefer lower pH, as 4 (Shockey and Shockey 2014).

As it can be understood, vegetable fermentation is a process where environmental conditions have not a specific recipe but they are a determining factor for the success of fermentation.

5. Spices and condiments

When vegetables are fermented, various seasonings can be added to fermentation such as red pepper powder, garlic, ginger, green onion, fermented seafood and many others. On last years, many efforts have been made to discover the right influence of these condiments during fermentation. Kimchi and sauerkraut fermentations are the two types of fermented cabbage that have been studied deeply since they are the most famous fermented cabbage in the world and they are going to be described below on section 11.

Red pepper powder is an emblematic ingredient of many Korean foods, like kimchi. Recent studies have demonstrated that fermentation of kimchi with red pepper powder is slower than the one without this condiment which was demonstrated for the decrease of pH value during the first hour of fermentation (Jeong et al. 2013; Kang et al. 2016). For middle or late stages of fermentation, significant effects were not identified. Red pepper powder was also observed to strongly influence the density of particular lactic acid bacteria during kimchi fermentation. The proportion of *Weissella* was higher in kimchi with red pepper powder than in kimchi without this condiment and the proportion

of *Leuconostoc* and *Lactobacillus* species were lower in kimchi with red pepper powder (Jeong et al. 2013; Kang et al. 2015; Kang et al. 2016). These three species are the principals in vegetable fermentation and they will be described below on section 9 (Kang et al. 2015; Ono et al. 2015).

Garlic and algae were studied in sauerkraut formulations and the results showed that acidity was higher in the treatments with garlic and algae however, sensory evaluation showed that sauerkraut juices with garlic tasted better but with algae were not very appealing (Wiander and Palva 2011). Garlic has also antimicrobial properties against some kinds of bacteria and a strong antifungal activity (Saeed et al. 2016). Another study of sauerkraut was made with aniseed, fennel seeds, caraway, dill, garlic and mint (Wiander and Korhonen 2011). Results showed that mint produces more lactic acid bacteria but they had a slower growth in garlic experiments.

Previous studies about spices and their influence on lactic acid bacteria were done showing diverse results about oregano, rosemary, sage, thyme, clove, cinnamon and basil and their essential oils. Oregano has an inhibitory effect against *L. plantarum* in concentrations between 4-8 g/L, however, this species can develop resistance to the inhibitory effect of the spice (Zaika and Kissinger 1981; Zaika et al. 1983). The effect of rosemary, sage and thyme were similar to the results of oregano but these spices were not as inhibitory since a concentration of 8 g/L allowed bacteria to survive and grew. The inhibitory activity is higher for oregano, then to rosemary and sage and finally to thyme (Zaika et al. 1983; Kozłowska et al. 2015; Saeed et al. 2016). In spite of these studies, lactic acid bacteria are generally less sensitive to spices than other Gram positive organisms and *L. plantarum* was found to be, most of the times, stimulated by natural spices such as cumin, rather than with their oleoresins (Shelef 1984; Kozłowska et al. 2015; Dunn et al. 2016).

Although all these particular effects that spices and condiments may have individually, the principal effects that have been reported is the inhibition of yeasts and molds (Shelef 1984; Wiander and Korhonen 2011; Wiander and Palva 2011; Katz 2012; Jeong et al. 2013).

6. Benefits of fermented vegetables

Fermented vegetables have numerous benefits for human body. Apart from maintaining probiotics in our diet, they also preserve vitamin C by slowing down its loss

(Katz 2012). Therefore, vitamin C is one of the benefits that this kind of fermented food has. Vitamin B_{12} is also present in fermented vegetables which is a good reason to eat them, especially people on a strict vegetarian diet, since this vitamin is present on animal-based foods (Jägerstad et al. 2004; Shockey and Shockey 2014). Thus, fermentation preserves vegetables raw, without heat treatments, so it retains their vitamins, minerals and enzymes. It preserves B and C vitamins and it makes nutrients readily available (Lee 1997; Shockey and Shockey 2014).

Deeper studies on fermented vegetables have shown that there are much more benefits beyond what has already been said. Kimchi, for example, is a low caloric food (18 kcal/100 g) that contains high levels of vitamins and minerals (Na, Ca, K, Fe and P), dietary fiber (24 % on dry basis) and other functional components, such us phytochemicals that are active compounds that have demonstrated anti-cancer, anti-obesity and anti-atherosclerotic functions (Park et al. 2014).

Finally, this type of fermentation done with lactic acid bacteria, prevents the use of chemical food additives such as nitrite, sulphite, sorbic acid, among others, since these bacteria produces several natural antimicrobials, including organic acids (lactic acid, acetic acid, formic acid), carbon dioxide, hydrogen peroxide, ethanol and bacteriocins (Leroy and De Vuyst 2004).

7. Problems in fermentation

Normally, problems that can occur in vegetable fermentation are due to the life of this food, therefore, there are some situations that are common and happen with some frequency like contaminations, high salinity levels and soft vegetables.

A common problem that has already been noticed is the growth of undesired molds and yeasts in surface of fermented vegetables. These contaminants should be removed and fermented vegetables should be safe under the brine (Katz 2012; Shockey and Shockey 2014).

When vegetables are over-salted, the common action is adding water and mix until the salinity level reach normal values or rinse vegetables before eating, however, this solution can throw away nutrients and bacteria (Shockey and Shockey 2014).

Vegetables with a soft texture can be reached by a quick fermentation, insufficient salt strength and air pockets. These vegetables are safe to eat, however, most of people

prefer eating them crunchy. To inhibit this problem, lower temperatures and higher salinity should be applied (Katz 2012; Shockey and Shockey 2014).

8. Lactic acid fermentation

Lactic acid fermentation represents the easiest and the most valuable biotechnology to keep and enhance the safety, nutritional value, sensory and shelf life properties of vegetables. Therefore, this fermentation constitutes the most suitable way for increasing the consumption of fresh-like vegetables (Di Cagno et al. 2013; Montet et al. 2014).

Bacteria which are responsible for the production of lactic acid can be divided into two sub-groups: homo-fermentative and hetero-fermentative. The first ones only produce lactic acid through the glycolytic pathway, so lactate dehydrogenase transforms two molecules of pyruvate into two molecules of lactic acid (Lahtinen et al. 2012; Montet et al. 2014). Hetero-fermentative bacteria produce not only lactic acid but also ethanol, acetate and carbon dioxide through 6-phosphogluconate/ phosphoketolase pathway (Jung et al. 2014; Montet et al. 2014). Hetero-fermentative bacteria are also capable of using fructose as an electron acceptor, converting it to mannitol (Mozzi et al. 2010; Jung et al. 2014; McFeeters et al. 2013). For a better understanding, **Figure 1** represents a proposed pathway for glucose metabolism of hetero-fermentative lactic acid bacteria.

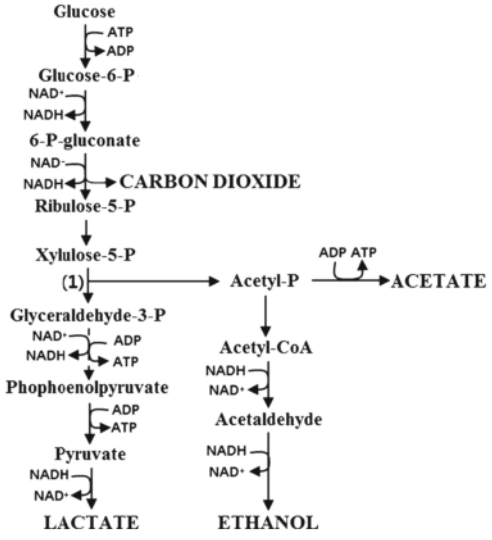

Figure 1 – A proposed pathway for glucose metabolism of hetero-fermentative lactic acid bacteria during kimchi fermentation. Adapted from Jung et al. (2014).

Both metabolic pathways are common in vegetable fermentation but their incidence depends on what kind of microorganisms are present on the fermentation medium.

9. Microorganisms involved in lactic acid fermentation

Lactic acid bacteria (LAB) are the responsible for the desired fermentation on vegetables. They are Gram positive, non-sporulating, catalase-negative, acid-tolerant and facultative anaerobic organisms (Mozzi et al. 2010). Depending on the species of vegetables, there are different studies from which it was possible to isolate different autochthonous microorganisms. Recently, molecular methods, such as microarrays, denaturing gradient gel electrophoresis and 16S sequencing, had been used to study fermentation of vegetables, as kimchi, and complex microbiota was revealed. A variety of *Weissella*, *Leuconostoc*, *Pediococcus* and *Lactobacillus* species, as well as yeasts and Archaea, have been identified as the principal species associated with vegetable fermentation (Daeschel and Fleming 1984; McFeeters et al. 2013). Their characteristics are described in **Table 1.**

Table 1 – Characteristics of LAB associated with vegetable fermentation. Adapted from Daeschel and Fleming (1984).

	Lactobacillus plantarum	***Lactobacillus brevis***	***Pediococcus pentosaceus***	***Leuconostoc mesenteroides***
Morphology	Short to medium rods usually singly	Short rods, occurring singly or in short chains	Cocci occurring singly, in pairs and in tetrads	Cocco or bacilli usually in pairs
Optimum temperature	30-35 ºC	30 ºC	35 ºC	20-30 ºC
Growth at 45 ºC	No	No	Yes	No
Growth in 8% NaCl	Yes	No	Yes	No
Glucose Metabolism	Homo Fermentative	Hetero Fermentative	Homo Fermentative	Hetero Fermentative

LAB are naturally present in raw materials and their population constitutes a small part of the autochthonous microbiota of raw vegetables, about 2.0-4.0 log cfu g^{-1}, in a total microbial population between 5.0-7.0 log cfu g^{-1} (Di Cagno et al. 2013). **Table 2** shows different species of lactic acid bacteria and their source.

Table 2 – Species of lactic acid bacteria, which were isolated from raw or spontaneously fermented vegetables. Adapted from Di Cagno et al. (2013).

Lactic acid bacteria species	**Source**
Lactobacillus plantarum	Tomatoes, marrows, carrots, cucumbers, eggplants, red-beets, capers, fennels, cabbages
Lactobacillus pentosus	Capers, eggplants, cucumbers
Lactobacillus fermentum	French beans, red beets, capers, eggplants
Lactobacillus curvatus	Peppers
Lactobacillus brevis	Tomatoes, capers, eggplants, cabbages, cucumbers
Lactobacillus paraplantarum	Cabbages, capers
Leuconostoc mesenteroides	White cabbages, carrots, peppers, cucumbers, eggplants, lettuce
Weissella soli	Carrots
Weissella confusa *Weissella cibaria*	Peppers, tomatoes
Enterococcus faecalis *Enterococcus faecium*	French beans, tomatoes, capers
Pediococcus pentosaceus	French beans, tomatoes, cucumbers, capers, cabbages

The most frequent species observed in the fermentation process are *Lactobacillus plantarum, Lactobacillus brevis* and *Leuconostoc mesenteroides*. However, this fact does not mean that these species will have a significant amount on fermentation. Actually, fermentation can have different types of microorganisms depending on the vegetables that are going to be used, environmental conditions and the growth of microorganisms, not only by spontaneous fermentation but also by the use of starter cultures. For example, studies on the classic sauerkraut showed that there is a sequence of lactic acid bacteria in fermentation. Firstly, *Leuconostoc mesenteroides* grows producing lactic and acetic acids and carbon dioxide which flushes out the oxygen. Then, *Lactobacillus brevis* grows acidifying the medium and finally, *Lactobacillus plantarum* grows producing more acid and lowering pH to below 4.0 (Steinkraus 2002; Xiong et al. 2012). By the same way, recent studies on kimchi production showed that, during early fermentation, the unclassified groups of microorganisms was very high, 97 %, and then it started to decrease during the rest of fermentation, what it is supposed to happen. Moreover, while genus *Weissella* was identified but remained approximately constant, genus *Leuconostoc* was the most abundant throughout fermentation. Genus *Lactobacillus* increased after the first stage of fermentation, which are similar results obtained for sauerkraut (Di Cagno et

al. 2013). These results are presented in **Figure 2** in order to have detailed information about time, pH and relative abundance of microorganisms.

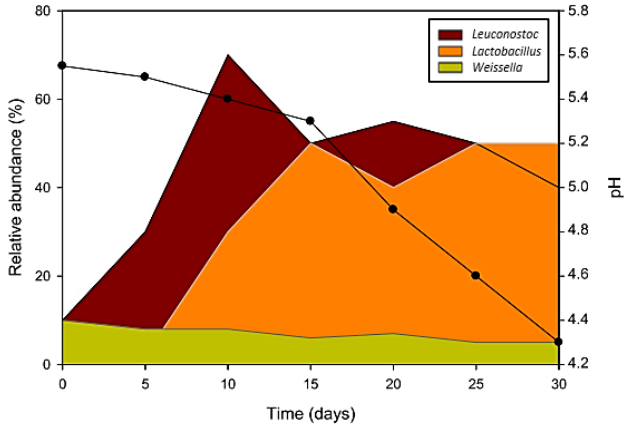

Figure 2 – Succession of lactic acid bacteria and change of pH during fermentation of kimchi. Adapted from Di Cagno et al. (2013).

Spontaneous fermentation occurs when the autochthonous microbiota of raw vegetables is used. Thus, providing favourable conditions of anaerobiosis, water activity, salt concentration and temperature, spontaneous fermentation starts. Notwithstanding the reliable value of this fermentation, there are some factors that are in favour of the use of starter cultures such as the risk of fermentation failure, the incorrect inhibition of spoilage and pathogen microorganism and the unpredictable variations of the final product properties (Di Cagno et al. 2013).

The optimization of fermentation can also be done through backslopping. This technique refers to the inoculation of the raw material with a small quantity of a previously performed successful fermentation, which has the best adapted strains. Backslopping represents a cheap and reliable preservation method and it is specially used when the microbial ecology and the role of successions in microbial population are not well known (Leroy and De Vuyst 2004).

Once quality control is essential for the industrialization of fermenting processes, the use of starter cultures in vegetable and fruits is increasing (Di Cagno et al. 2013; Montet et al. 2014). The main options to control lactic acid fermentation are the use of autochthonous or allochthonous starters. Autochthonous means isolated from the same raw matrix, and re-used on the same matrix, and allochthonous means isolated from certain raw matrices but used to ferment different products. This last type of starters are

the ones used in commercial starters, since they are used to ferment a variety of vegetables and fruits (Di Cagno et al. 2013; Yuliana et al. 2013; Montet et al. 2014). Normally, the activity of commercial cultures inhibit spoilage microorganisms and non-LAB (Yuliana et al. 2013), prevent softening and stabilize the natural colours of vegetables. However, this kind of starters have some limitations such as the fact that selection time only considers rapid acidification, the adaptation to the matrix properties be poor and its diversity do not reflect the ecosystem where they have to be used (Leroy and De Vuyst 2004; Di Cagno et al. 2013). In order to study starter cultures, the main criteria for its selection are the rate of growth, the rate of total production of acids, which affects the changes of pH, and the environmental adaptation/tolerance, which englobes temperature, degree of exposure to air, concentration of fermented carbohydrates, buffering capacity, pH and presence of naturally inhibitory compounds in raw vegetables (Di Cagno et al. 2008). Despite the studies and the lists of microorganisms with certified use in food fermentations (Bourdichon et al. 2012), to guarantee industrial scale of vegetables fermentation, commercial starters with high performances and well-studied are needed. Autochthonous microbiota should be selected as starter culture since these cultures may ensure prolonged shelf life and more targeted nutritional and sensory properties.

10. Equipment used in vegetable fermentation

Fermentation of vegetables is an ancient tradition and nowadays it still remains mainly a homemade product due to its process simplicity. There are specific tools that people usually use in this submerged fermentation such as glass jars, ceramic crocks and airlock systems.

Glass jars are usually the equipments that are used when people start to ferment because they are cheap, it is possible to watch and control what is happening during the fermentation and it is possible to do small batches. However, this equipment has some limitations since it needs special attention to maintain vegetables submerged, to release carbon dioxide from inside and to prevent direct light (Katz 2012; Shockey and Shockey 2014). To solve this problem there is an airlock system which is affixed to the lid of a jar, keeping new air out of fermentation and releasing carbon dioxide produced inside. Thus, the major disadvantage of jars is the limited of vegetables that can be fermented (Katz 2012; Shockey and Shockey 2014).

Ceramic crocks allow larger fermentations than the jars and they keep the fermented vegetables in a cold and dark environment. Water-seal crocks are one type of

useful crocks that have an outer deep rim with a trough that holds water and when the lid is placed in the trough, water creates an airlock. Lid has a small hole in the rim that allows carbon dioxide to escape without allowing air into the crock. Thus, this equipment has already a solution for the carbon dioxide production and for submersion of vegetables (Shockey and Shockey 2014). There are more traditional equipments but these ones are the most used and useful. **Figure 3** shows the equipments that were described above.

Figure 3 – Equipments used for vegetables fermentation. 1 – Glass jars with an airlock system. 2 – Water-seal crocks. 3 – Upper part of a water-seal crock with the weight inside. Adapted from Shockey and Shockey (2014).

The scale up of the fermentation process is normally the final step in any research and development project. Large-scale fermentations are usually carried out in stainless-steel tank fermenters which must allow an easy way to sterilize, operate, monitor and take samples (Soccol, Pandey, and Larroche 2013). The bioreactor is probably the most important part of the process since it is there that all the fermentation occurs. Pilot and large scale bioreactors can be found in innumerous companies such as B. Braun Biotech-Sartorious (Germany), Eppendorf (USA), Applikon (Netherlands) and Bioengineering (Switzerland) (Soccol et al. 2013).

11. Types of fermented vegetables

There is no known vegetable that cannot be fermented since the process of vegetable fermentation is extremely versatile. Notwithstanding, there are vegetables more suitable for being fermented that taste better and do not get mushy so fast.

Nowadays, the lactic acid fermentation of vegetables has an industrial significance mainly for cabbages, cucumbers and olives. Examples of fermented vegetables manufactured in various worldwide regions are presented in **Table 3**.

Table 3 – Examples of emerging and traditional fermented vegetables which are manufactured in different worldwide regions. Adapted from Di Cagno et al. (2013).

Product	**Main ingredients**	**Worldwide place**
Sauerkraut	Cabbage, salt	Europe, USA
Cucumbers	Cucumbers, vinegar, salt	USA, Asia
Capers	Capers, water, salt	Mediterranean Countries (Greece, Italy, Spain
Kimchi	Cabbage, radish, salt, spices and other vegetables (ginger, pepper, garlic, onion)	Korea
Sinki	Radish roots	Eastern Himalaya
Khalpi	Cucumber	Eastern Himalaya

Commercial production of fermented cabbage comprises kimchi, made from the Chinese cabbage, and sauerkraut, from *Brassica oleracea*, in United States (US) and Europe, respectively.

Sauerkraut fermentations are usually done in bulk fermentations tanks that may contain hundreds of tons of shredded or chopped cabbage. This cabbage is dry-salted as it is conveyed to fermentation tanks and a brine solution is formed in fermenting tanks with a NaCl concentration of about 2-3% (Daeschel and Fleming 1984; McFeeters et al. 2013). During the first hours, carbon dioxide, lactic and acetic acids produced by hetero-fermentative bacteria. After about one week of fermentation, hetero-fermentative bacteria are replaced by homo-fermentative bacteria who are more acid-tolerant. Sauerkraut can be obtained with high quality, without using starter cultures, through salt concentrations of about 2 % and temperatures near 18 ºC. Most of manufactures of US consider that sauerkraut may be stored for up one year in fermentation tanks until the final processes for food service and retail sale. However, this fermented vegetable may become too much sour with acid lactic accumulation. European manufactures usually pack sauerkraut at the end of heterolactic fermentation stage to produce a mild acid flavour product (McFeeters et al. 2013).

Kimchi fermentation is very similar with sauerkraut but in this fermentation the addition of other ingredients is common. Chinese cabbages are firstly cut and soaked in

brine with 5-10% in NaCl. Then they are washed and drained to mix them with other ingredients such as red pepper, garlic, ginger and green onion. Kimchi is prepared commercially or by individuals through the use of house-hold kimchi refrigerators. These equipments are small but they have a programmable-temperature that provides an initial temperature about 18 ºC for the first days followed by very cold refrigeration to values of about 1-2 ºC. All this process allows the initial hetero-fermentative bacteria to work but delays the homolactic stage of fermentation, keeping kimchi from becoming too sour. When pH and acidity reach values about 4.0 and 0.5 %, respectively, the optimum taste is attained (McFeeters et al. 2013).

Cucumber fermentations are a little different from cabbage. In the US, they are commonly fermented in 30 – 40 thousand liters, open-top and fiberglass tanks, located outdoor with the brine surface exposed to sunlight (Daeschel and Fleming 1984). The UV radiation kills aerobic surface yeasts. Generally, fermentations occur in a brine at about 6% NaCl (Daeschel and Fleming 1984) and calcium chloride (about 0.2 %) is added to cover brine in order to maintain crisp texture during fermentation and storage. This fermentation typically undergoes a homolactic acid fermentation and, at the end, cucumbers are stored in fermentation tanks for about 1 year or more. Prior to sale, these vegetables are washed to remove the excess of salt and packed with an appropriate cover liquid which contains acetic acid and spices (McFeeters et al. 2013).

Olive fermentations are the most different processes. To have a general idea, theses fermentations will depend on what kind of olives are going to be fermented. Green table olives are treated with NaOH solution, washed and fermented and black olives are slowly fermented without any treatment. Olives are brined in 10% NaCl and due to the initial pH of about 7, there are more microorganisms on the medium than for the others fermentations of cabbage and cucumbers (McFeeters et al. 2013). Finally, pepper is another vegetable that can be fermented by lactic acid bacteria. This fermented vegetable is not as famous as the previous ones but it is widely used in Portugal on red pepper paste formulations and its main purpose is food tempering (Koffi-Nevry et al. 2012). This topic is going to be described on sub-section 11.1 (Red pepper paste).

11.1. Red pepper paste

Red pepper paste is widely used for colouring and as a flavour ingredient in many countries. It is a traditional food that can be prepared in many different ways depending on the tradition behind. Gochujang and the turkish pepper paste are the most well-known

pepper pastes that have been studied in more detail (Kuleaşan and Okur 2012; Kwon et al. 2015). Portuguese pepper paste differs from the previous ones because it has not more ingredients in addition to pepper, like gochujang. Moreover, it is not processed with heat treatments, like the Turkish pepper paste.

Portuguese red pepper paste started to be a homemade product through a fermentation process in which pepper is mixed with salt and left in a closed container during days. After this process, the previous mixture is crushed and the resulting product is a salty homogeneous paste, good for tempering food.

Industrially, red pepper paste production is a widely known process carried out in many different ways. However, this product have been made from several years without any significant improvement. Efficiency may be improved and lower costs may be reached if deeper studies are carried out.

12. Pepper characteristics

Pepper belongs to the *Capsicum* genus, which is native from tropical regions of America, and it was brought to Portugal and Europe on the age of discoveries (Ferrão 1992; De Krishna 2003; Bosland and Votava 2012). Nowadays, pepper is produced in greenhouses, during the cold seasons, and outdoors during spring, being harvested from June until October.

It is still difficult to characterize peppers due to the various colours, sizes and phenotypes however, various authors consider 25-32 as the interval number of existent species for the *Capsicum* genus (Bosland and Votava 2012). The most famous are *C. annuum* (with Jalapeno and Bell varieties), *C. frutescens* (with Tabasco variety), *C. chinense* (Habanero varieties), *C.baccatum* and *C. pubescens* (De Krishna 2003; Bosland and Votava 2012). In spite of the variety that exists inside *Capsicum* genus, the major cultivated fields of pepper in the world normally belong to *C. annuum* specie. Regarding pod types, it is possible to distinguish different peppers which are normally called for commercial purposes. Bell group is probably the most economically important pod type which have the largest number of cultivars. Bell peppers can also be called California wonder, with a square shape, or La Muyo, with an elongated shape. (Bosland and Votava 2012). Industrially, California peppers are the most used due to its shape and fleshy properties.

Chemically, peppers are mainly constituted by water, fixed and volatile oils, carotenoids, capsaicinoids, protein, fiber and mineral elements. All the nutritional value,

taste, color and aroma are influenced by these constituents, especially by carotenoids that contribute for the color and nutritional value and by capsaicinoids, which are the constituents that give the pungent effect to hot peppers. Depending on the age and type of pepper, the amount and type of chemical constituents change (Bosland and Votava 2012).

MATERIAL AND METHODS

Several lactic acid fermentations of red pepper were monitored and evaluated. During all the fermentations, physical, chemical and microbiological parameters were determined and analysed in order to ascertain their changes during the course of fermentation.

1. Raw material

All red peppers used in the present work were from Almeria (Spain) and were bought to a local supplier. Red peppers belong to California commercial variety.

Initially, peppers were washed with tap water and all the stems were took out. Then, the vegetables were shredded in an industrial centrifugal mill (Pivoting centrifugal mill RM 2,2, Voran maschinen) and a small granulometry was obtained.

2. Fermentations

All fermentations were performed in order to study different parameters such as temperature (Friocell Incubator, MMM Group), salt content (Refined dried fine salt, Jumsal) and the use of microflora, two pure starter cultures and backslopping. Pure cultures of *Leuconostoc mesenteroides* 001 and *Lactobacillus plantarum* 017 were supplied by Mendes Gonçalves' culture collection and these bacteria were preserved on skim milk 10% in a concentration of $1{,}56\times10^{10}$ cell/mL and 2×10^{10} cell/mL, respectively. They were inoculated together in fermentations at a total cell concentration of 10^6 cell/mL each, as previously described by diverse authors (Špička et al. 2002; Yuliana et al. 2013; Xiong et al. 2014).

In order to maintain the anaerobic conditions necessary for the lactic acid fermentations, essentially to prevent moulds development, an airlock was put in all containers where fermentations took place. All the samples were taken out from a tap on the bottom of the container and before each fermentation all containers were washed with diluted sodium hypochlorite (10% w/v).

Fermentations were analyzed through samples taken every 12 hours which were divided in three falcon tubes: one for pH and titratable acidity analysis, one for HPLC analysis and one for identification and quantification of lactic acid bacteria.

Fermentations finished when pH reached constant values for two or three measures or reached pH around 3.5.

At the end of fermentations, rep pepper paste was produced according to a Mendes Gonçalves recipe.

2.1. Temperature

The temperatures that were performed in these experiments were 18 °C and 29 °C and fermentations were carried out with the use of mixed starter cultures described above and the microflora present in red pepper. For each fermentation, shredded red pepper (4 kg) was mixed with 2% of salt and, in case of using starter cultures, pure cultures were added to fermentation with the concentration said before.

2.2. Salt content

In order to study the influence of salt content on lactic acid fermentation, three fermentations were done with 0%, 2% and 4% of salt using natural red pepper microflora at 29 °C.

2.3. Backslopping

Backslopping was tested at 29 °C in red pepper fermentations with starters and with autochthonous microflora. Salt was used at 2%.

An initial fermentation was done with a mixed starter culture of *Leuconostoc mesenteroides* and *Lactobacillus plantarum.* In order to have an initial total cell concentration of 10^6 cell/mL for the next fermentation, 1% of the previous whey was inoculated to the next fermentation. A percentage of 10% was also studied. This procedure was done three and two times for 1% and 10%, respectively, as it can be seen in **Figure 4**. In case of fermentations carried out with red pepper microflora, the same procedure was done, testing two times 1% of the previous fermentation serum.

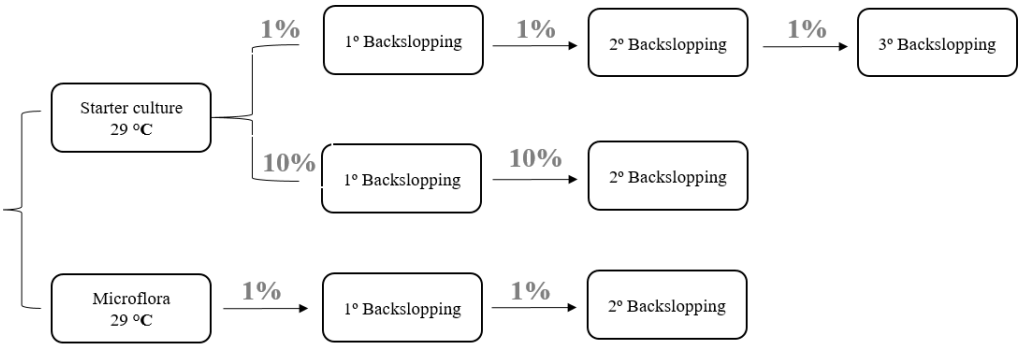

Figure 4 – Fermentation scheme of backslopping procedure.

2.4. Commercial mixed starter culture

An initial commercial starter culture produced by Cutting Edge Cultures in USA was evaluated. It is constituted by *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Pediococcus acidilactici* and organic tapioca sugar. Initially, the content of one sachet was hydrated in 235 mL of distillate water for 10 minutes and then it was joined to 2,3 kg of shredded red pepper with 2% of salt. The temperature used for this experiment was 29 °C.

2.5.Growth curves

Firstly, temperatures of 60 °C, 70 °C and 90 °C were tested during 10 minutes on shredded red pepper in order to study the existence of lactic acid bacteria after the heating process. Samples were collected after the heating and the microbial load of the samples was quantified in terms of microorganisms as explained below in sub-section 3.3 (Total viable lactic acid bacteria quantification).

In order to study growth curves of *Leuconostoc mesenteroides* and *Lactobacillus plantarum*, shredded red pepper with 2% of salt was heated at 70 °C during 10 minutes. Hot red pepper was packed on the fermentation container, previously washed and disinfected with diluted hypochlorite (10% w/v), and cooled inside it. Then, starter cultures of *Leuconostoc mesenteroides* and *Lactobacillus plantarum* were inoculated separately. It was also tested the fermentation without any inoculation. Fermentations were carried out at 29 °C.

3. Analyses to evaluate fermentation

3.1. pH

The pH was measured with a pH meter (Hanna Instruments 2002-02) equipped with a glass electrode calibrated on sampling days using standard buffer solutions (Hanna Instruments) at pH 4.01 and 7.01. Values were took two times and the mean value as well as standard deviation were calculated.

3.2. Titratable acidity

Titratable acidity (TitroLine easy from Cetotec) was measured using 1M NaOH and a pH meter calibrated on the sampling days using standard buffer solutions (Hanna Instruments) at pH 4.01 and 7.01. Values were took two times and the mean value as well as standard deviation were calculated.

3.3. Total viable lactic acid bacteria quantification

Lactic acid bacteria were quantified after the collection of fermentation samples which were stored at 3.5 ± 1 °C until microbial analysis (maximum one or two days of storage).

Samples were diluted from 10^{-4} to 10^{-9} with 0.1% of buffered peptone water (Scharlau Chemicals) and, depending on the fermentation stage, LAB were cultivated from 10^{-4} to 10^{-6}, for the first stages, and from 10^{-6} to 10^{-9} for the last stages.

The culture media for the quantification of LAB was MRS (deMan, Rogosa and Sharpe, Frilabo) and these bacteria grow up during 48 – 72h at 30 ± 1 °C (Cho et al. 2006; Di Cagno et al. 2008; Di Cagno et al. 2009; Alberto et al. 2013). MRS was autoclaved at 121 °C for 15 minutes. The microorganisms were cultivated, in duplicate for each dilution, using the pour-plate method (Tamang et al. 2005; Saeedi et al. 2015) which consists on place 1 mL of sample of each dilution into a sterile Petri dish and then place sterile molten MRS and mix well. At the end, plates were incubated at 30 ± 1 °C during 48 – 72h, as referred before.

After incubation, colony count was done according to the general ranges for countable numbers of colonies on a plate, 30 – 300 colonies. Then, these numbers were converted to Colony Forming Units (CFU) per mL of sample. The final value was obtained from the mean value of all dilutions and their duplicates and standard deviation was also calculated.

3.4. HPLC analysis

Lactic and acetic acids were identified and quantified by HPLC-UV/Vis. Organic acids were extracted from the samples and they were preserved until HPLC analyses. All the procedure was based on Meireles (2012).

3.4.1. Extraction

Initially, 12.5 mL of 0.4% ortho-phosphoric acid (H_3PO_4 85% (Carlo Erra)), at pH 2.10 (Hanna Instruments 2020) were added to 2 mL of fermentation sample. To homogenise, solutions were stirred during 4 minutes with a vortex and after they were centrifuged at 4 °C (5810 R Eppendorf) for 40 min at 3100 g. The resulting supernatant was filtered through a membrane filter of nylon with a 0.45 µm pore (VWR) for 2 mL vials (VWR). After this liquid-liquid extraction, samples were preserved in the freezer at -15 °C until HPLC analysis.

3.4.2. Identification and quantification by HPLC UV/Vis

Organic acids attempted to be identified and quantified by HPLC UV/Vis (Andersson and Hedlund 1983; Seydim et al. 2000; Zeppa et al. 2001; Meireles 2012; Alberto et al. 2013)

Standards of lactic and acetic acids were prepared in order to validate a calibration curve obtained by Meireles (2012), present on **Annex A.1**. Initial solutions in a concentration of 0.1 g/L of lactic acid (Sigma-Aldrich) and acetic acid (VWR) were prepared in order to prepare other two standard solutions of these acids in a concentration of 5×10^{-2} and 1×10^{-2} g/L. Composed standards were also prepared (1 mL of sample + 1 mL of 0,1 g/L lactic or acetic acids) in order to verify the presence of the analysed acids. All of the standards were filtered through a membrane filter of nylon with a 0.45 µm pore (VWR) for 2 mL vials.

The mobile phase used for this HPLC analysis was 0,4% ortho-phosphoric acid (Cho et al. 2006), the same solution used on the extraction process. Mobile phase was also filtered through a membrane filter of nylon with a 0.45 µm pore (VWR).

On the HPLC equipment, each standard solution/sample was injected twice (20 µL each) with a running time of 30 min and with a flow rate of 0.7 mL/min. The column worked at a temperature of 25 °C and the wave length of detection was 210 nm (Cho et al. 2006). Equipment characteristics are presented in **Table 4**.

Table 4 – Characteristics of HPLC equipment.

Column	C_{18} (Merck, LiChroCART ® 250-4 Purospher ® STAR RP-18 endcapped (5 µm))
Oven	Jasco CO-2060 Plus
Injector	Jasco AS-2057 Plus
Detector	UV-Vis (Jasco MD-2515 Plus)
Pump	Jasco PU-2080 Plus
Ternary Gradient Unit	Jasco LG-2080-02
Degasifier	Jasco DG-1580-54
Software	Jasco ChromPass Chromatography Data System 1.8.2.1

Between every two sample analysis, a column cleaning process was carried out through a gradient of mobile phase and methanol. Initially, mobile phase passed through the column for 5 min. Then, during 1 min, the passage of mobile phase to methanol which was left running for 5 min was performed. Finally, the passage of methanol to mobile phase during 1 min was performed and mobile phase was left running for 5 min.

4. Sensory analysis

This work also intend to evaluate the final products obtained and correlate their organoleptic characteristics with the physical-chemical and microbiological parameters involved in their production.

Sensorial analysis was done by some members of Research and Development department of the company, a group of 9 non-expert tasters of both genders and different ages (23 – 40). A discriminative test was done to all elements (triangle test) in order to ascertain the sensory differences between two products. After this, volunteers who realised the difference on triangle test answered an affective test to choose which product they liked the most.

Evaluated red pepper pastes were tasted in a proportion of 15 g of pepper paste to 100 g of meat steaks and cooked similarly.

5. Statistical analysis

Differences between samples were statistically studied through the use of Student's t-test of Microsoft Excel 2013. To reject the null hypothesis, the value of p was set to < 0.05.

RESULTS AND DISCUSSION

In this section, all the different conditions studied on each fermentation such as temperature, salt content, microflora, pure starter cultures, commercial starter cultures and backslopping, are going to be discussed separately. It is important to note that information about the products of fermentation are not present due to the fact that data from HPLC were not taken into account due to problems on the equipment as it is shown on **Annex A.2**.

1. Temperature

Two temperatures, 18 and 29 ºC, were evaluated with the use of pepper microflora and mixed starter cultures.

Titratable acidity and pH of fermentations carried out at 18 ºC were analysed and the results are presented in **Figure 5**. Fermentations were done in duplicate, two using pepper microflora and two using a mixed starter culture.

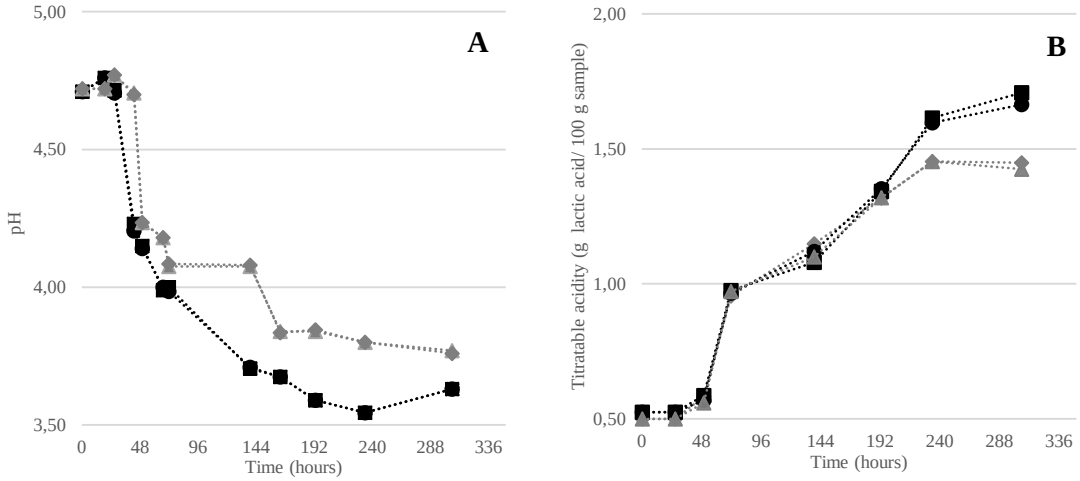

Figure 5 – pH (A) and titratable acidity (B) results of fermentation at 18 °C. Microflora is represented by M1 (▲) and M2 (◆) and mixed starter culture is represented by S1 (■) and S2 (●). Points represent average and error bars the standard deviation.

There are general conclusions that can be taken from the previous results at 18 ºC. Firstly, fermentations were done in duplicate and results show that both values of pH and titratable acidity are very similar during all the process. Differences between S1 and S2 and between M1 and M2 were statistically studied and they were not significantly different according to the method adopted to accept the null hypothesis, which means that

$p > 0.05$. This similarity between values from equal fermentations happened two times, one with the present situation and one with an initial fermentation performed at 22 °C with a wrong pure starter culture. These data were not presented as it has no interest for the purpose of this work. However, due to this similarity on both fermentations, it was decided to stop with the repetition of fermentations with the same conditions in order to evaluate the maximum variables possible. Secondly, fermentations using initial starter cultures reached lower pH values and higher acidity levels. This can be explained by the fact that fermentations using initial starter cultures had a higher concentration of LAB than the ones using just pepper microflora. The results appeared quickly and efficiently as it was described in literature review section. In fact, starter cultures increase the concentration of LAB and inhibit the growth of undesired microorganisms (Stamer et al. 1971; Daeschel and Fleming 1984; Halász et al. 1999; Leroy and De Vuyst 2004). Finally, at 18 ºC, data from pH but especially from acidity seem to have two phases where the values change at different rates. The first one until nearly 70 hours of fermentation and the second one after this time. This fact may have happened due to the natural succession that LAB have on these kind of fermentations however no conclusions can be made about this topic (Steinkraus 2002; Xiong et al. 2012; Di Cagno et al. 2013).

At 29 ºC, fermentation reached a lower pH (3.40) and higher acidity levels (1.8 – 2 %) in less time compared with the one at 18 ºC, which is normal as bacteria are in the optimal temperature to grow, as shown in **Table 1** (section 9 of Literature Review).

The results of pH and titratable acidity of fermentations carried out at 29 °C are presented in **Figure 6**.

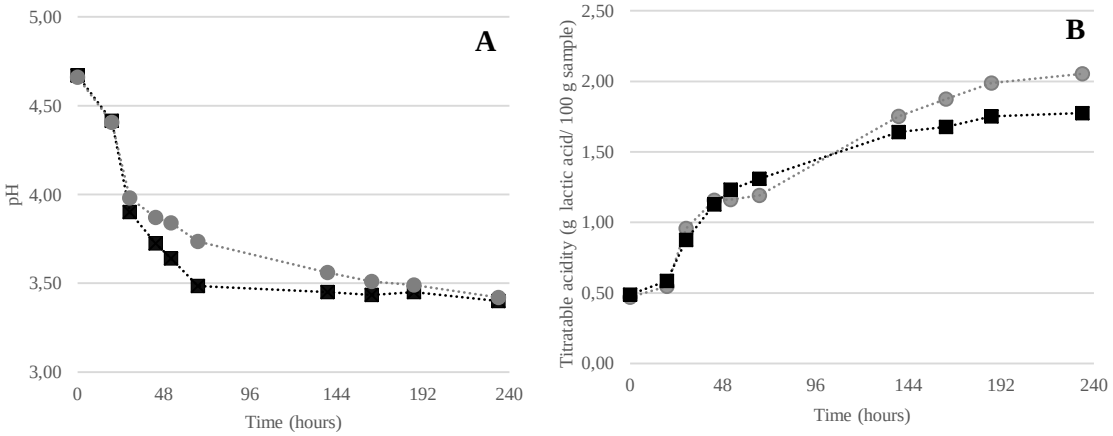

Figure 6 - pH (A) and titratable acidity (B) results of fermentation at 29 °C. Microflora is represented by M1 (●) and mixed starter culture is represented by S1 (■).Points represent average and error bars the standard deviation.

Comparing to what happened at 18 ºC, at 29 ºC the pH was lower and acidity was higher for both fermentations where microflora and starter cultures were used. This may be caused by the influence of the temperature on their metabolism. LAB grow faster at temperatures nearly 30 °C and they are able to produce more metabolites, increasing the acidity to levels that on lower temperatures they are not able to do, at least on the period of time that was studied (Halász et al. 1999; Alberto et al. 2013). Furthermore, at 29 ºC, fermentation that used microflora reached higher values of acidity than the one that used starter cultures. This fermentation was always decreasing its pH and increasing acidity which may be caused by the natural development and diversity of LAB at the final stages of fermentation.

Regarding pH and acidity data from both fermentations in **Figure 6**, it is also possible to found two different phases. The first one until nearly 48 hours and the second one after this time. As well as explained for the fermentation that was carried out at 18 °C, the natural succession that LAB have on these kind of fermentations may explain these two different rates on pH decrease and acidity increase.

Likewise, data from total viable LAB quantification are in agreement with what was said before about pH and acidity results at 18 and 29 °C. Total viable LAB data are presented in **Figure 7**.

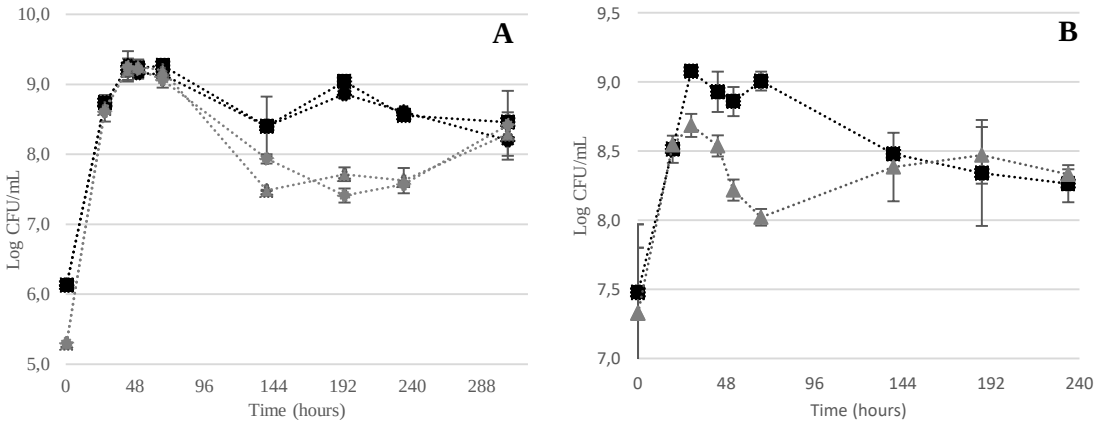

Figure 7 - Total viable LAB quantification results of fermentation at 18 °C (A) and at 29 ºC (B). Microflora is represented by M1 (▲) and M2 (◆) and mixed starter culture is represented by S1 (■) and S2 (●).Points represent average and error bars the standard deviation.

At 18 ºC (**Figure 7A)** it is possible to observe the similarity between the values of total viable LAB that was observed previously for pH and acidity results for the same temperature (p > 0,05). Fermentations inoculated with initial starter cultures revealed

higher values of total viable LAB, which may justify the lower values of pH and higher values of acidity that were reached. It is also possible to observe that LAB had two phases of growth, the first one until nearly 70 hours of fermentation and the second one after this value (**Figure 7A)**. These periods of time are the same that were described when discussing pH and acidity data at 18 °C, which may suggest that the changes on the medium properties are strictly linked with the number of total LAB. However, it is not possible to correlate directly that information with the characteristic microorganisms' successions on lactic acid fermentations since they are not conclusive as it was already said about pH and acidity results.

At 29 ºC, data of total viable LAB presented in **Figure 7B** are also in agreement with what happened with pH and acidity values as the number of LAB was higher for fermentations with starter cultures. At the final stages, the number of LAB from fermentation with microflora was higher than the number of LAB of fermentation with starter cultures which may also justify the higher acidity on final stages of fermentation (**Figure 6B)**. As well as happened with fermentations carried out at 18 ºC, data from total viable LAB describe the two different phases of growth which are correlated with pH and acidity data in **Figure 6**. Firstly, until 48 hours of fermentation, there was a fast increase in the number of total viable LAB which is strictly linked with the decrease on pH and the increase on acidity. Then pH and acidity changed as well, but at lower rates.

Therefore, higher temperatures associated with the use of starter cultures may influence the behavior of bacteria. As it was discussed before, fermentations ended earlier because LAB were on a favourable temperature to grow and starter cultures gave an advance on the natural progression of fermentation (Stamer et al. 1971; Alberto et al. 2013).

Comparing the number of total viable LAB from both fermentations at different temperatures it is possible to realize that the number of LAB on fermentations using starters are almost always higher than the ones using microflora. Regarding the different stages of fermentations, on the beginning LAB are always on concentrations of 5 -7,5 CFU/mL, they were capable to reach a maximum concentration nearly 9 CFU/mL and, at the final stages of fermentation, they had concentrations between 8 – 8,5 CFU/mL.

It is important to note that microorganisms that can grow on MRS are not only lactic acid bacteria. Yeasts can also grow, and these values were supposed to be quantified with the identification of microorganisms proposed on the present thesis' objectives (De Man et al. 1960). Since this analysis was not performed, it is not possible to realize the

truly number of LAB. However, the number of undesired microorganisms was not taken into account since lactic acid fermentation conditions are supposed to inhibit them as it is described in the literature (Stamer et al. 1971; Lahtinen et al. 2012).

At the end of this experiment, a quickly sensory test between some members of R&D department was made to evaluate if the slow fermentation gave some special flavor to the final red pepper paste. It was concluded that differences between the two products were not relevant when applied, especially due to the high salt content that red pepper paste needs to have. Therefore, to proceed the present work, a temperature of 29 ºC was chosen to evaluate the other parameters as fermentation occurred faster at this temperature.

2. Salt

Salt content was studied at 29 ºC in order to understand its influence in lactic acid fermentation using pepper microflora. Percentages of 0, 2 and 4% of salt were studied and results of pH and titratable acidity are presented in **Figure 8**.

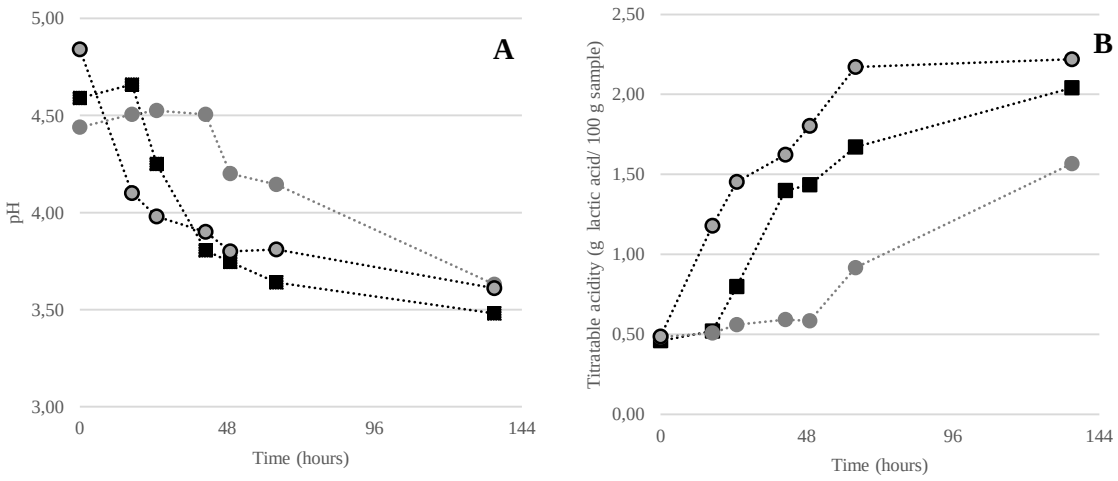

Figure 8 - pH (A) and titratable acidity (B) results of salt content study: 0 (●), 2 (■) and 4% (●). Points represent average and error bars the standard deviation.

From the data showed above (**Figure 8**), the principal effect of increasing the salt content is the delay of fermentation. Both pH and titratable acidity values show that fermentation is much more delayed when a percentage of 4% of salt is applied. Furthermore, fermentations without salt may start faster observing the initial decrease of

pH and increase of acidity, however, contaminations with other microorganisms, as yeasts and molds, may occur with the production of other types of products (Lahtinen et al. 2012; Katz 2012; Shockey and Shockey 2014).

Total viable lactic acid bacteria are in agreement with pH and acidity data since LAB from fermentation with 4% of salt just started to grow after 24 hours of the beginning of fermentation. In opposite, LAB from fermentation with 0% of salt started to grow immediately. Total viable LAB results are presented in **Figure 9**.

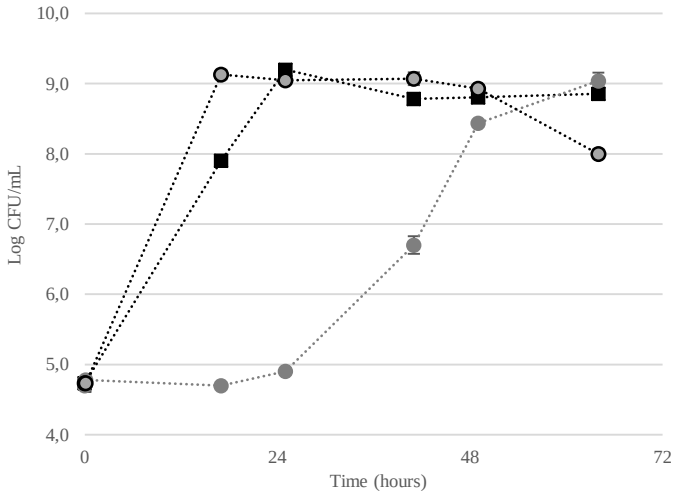

Figure 9 - Total viable LAB quantification results of salt content study: 0 (●), 2 (■) and 4% (●). Points represent average and error bars the standard deviation.

In these experiments, salt was only used for decreasing the probability of the growth of undesired bacteria. As the final product is red pepper paste, texture do not have such importance as for other vegetable fermentations, a fined refined salt was used to facilitate the process.

3. Commercial starter culture

In order to study the behavior of a commercial starter culture, three fermentations were carried out at 29 º C in which pepper microflora, mixed starter cultures and a commercial starter culture were studied. Results of pH and titratable acidity are presented in **Figure 10**.

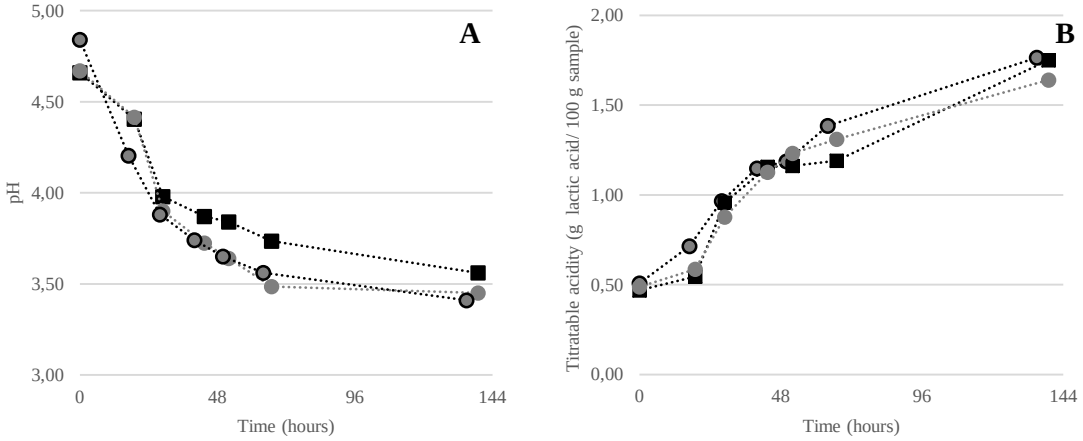

Figure 10 - pH (A) and titratable acidity (B) results of commercial starter culture study. Microflora is represented by (■), pure mixed starter cultures by (●) and Commercial starter culture by (◉). Points represent average and error bars the standard deviation.

The previous data show that all results of pure starter cultures with commercial starter cultures in terms of pH and acidity are very similar between them, without statistical significant differences (p > 0,05). This fact may suggest that starting a fermentation with pure cultures or with a commercial culture do not matter for the final conditions, they are reached equally and at the same time.

Comparing with the behavior of fermentation using microflora, the use of starter cultures have the advantage of reaching a pH of 3.5 in a faster and efficient way, nearly 72 hours, comparing with 144 hours. Regarding acidity, results are very similar for pure and commercial starter cultures and for microflora.

Commercial starter culture do not show the concentration at which bacteria are applied, it just says the quantity of powder to put in determined kilograms of vegetables. Therefore, with the quantification of total viable LAB of this fermentation, it is possible to realize that these bacteria have probably a lower concentration than 10^6 cell/mL comparing the values obtained for these three fermentations. On other way, the number of bacteria when starter cultures were used remained always higher than when they were not used, as it was already discussed for fermentations carried out at 18 and 29 °C.

Results of total viable LAB quantification are presented in **Figure 11**.

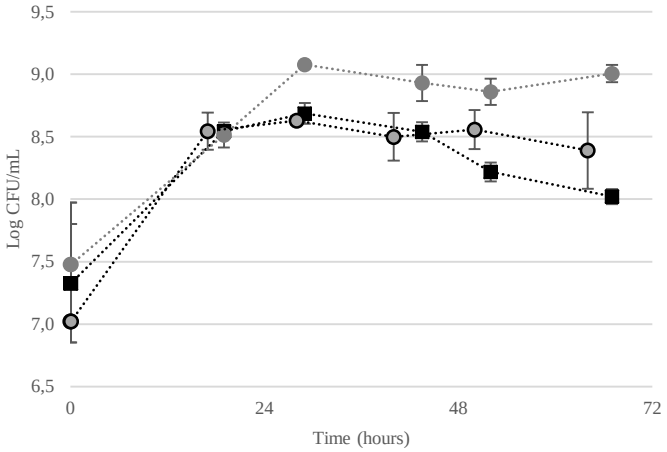

Figure 11 - Total viable LAB quantification results of commercial starter culture study. Microflora is represented by (■), pure mixed starter cultures by (●) and Commercial starter culture by (◘). Points represent average and error bars the standard deviation.

The use of starter cultures are important due to the control of microorganisms of fermentations, the standard products that can be obtained and the time of fermentations. However, on the present work, the fermented product that was intended to develop was red pepper paste which is not a value added product. Small flavours do not have an important role since red pepper paste have a strong salt content and, as it is only used as a tempering product, flavours from fermentation are very difficult to realize as it was proved when the small sensory evaluation was performed after fermentations carried out at different temperatures.

Therefore, improvements can be made on the existent process to reduce the time of fermentation and reach the same product parameters without increase the costs.

4. Backslopping

Backslopping represents a cheap and reliable preservation method which refers to the inoculation of a new fermentation with a previously successful fermentation (Leroy and De Vuyst 2004). In order to reduce the fermentation time and help it having the correct development, a percentage of a previous fermentation serum was introduced on a new fermentation. Firstly, 1 and 10% of serum from the fermentation using the pure starter cultures was used to start new fermentations. LAB were quantified on the serum in order to introduce the correct concentration of cells according to literature for this kind

of fermentations, 10^6 - 10^7 cell/mL (Špička et al. 2002; Yuliana et al. 2013; Xiong et al. 2014). The results obtained are presented in **Table 5.**

Table 5 – Concentration of LAB (cell/mL) used to start new fermentations through backslopping.

	1% Starter	**10% Starter**	**1% Microflora**
1º Backslopping	$5{,}25 \times 10^7$	$5{,}25 \times 10^7$	$2{,}15 \times 10^8$
2º Backslopping	$2{,}50 \times 10^7$	$8{,}58 \times 10^7$	$1{,}68 \times 10^8$
3º Backslopping	$5{,}70 \times 10^7$	-	-

According to the previous values, to have a final concentration of 10^6 - 10^7 cell/mL at the inoculum that is going to be introduced on a fermentation of nearly 4L, a total of 4 $\times 10^9$ cells is needed. As the serum have the concentrations of 10^7 – 10^8 cell/mL, to have that number of cells, it was needed 10^1 - 10^2 mL of serum, which corresponds to 1 and 10%, the percentage of backslopping that were used, respectively.

Results of the first backslopping of fermentation that used mixed cultures are presented in **Figure 12**.

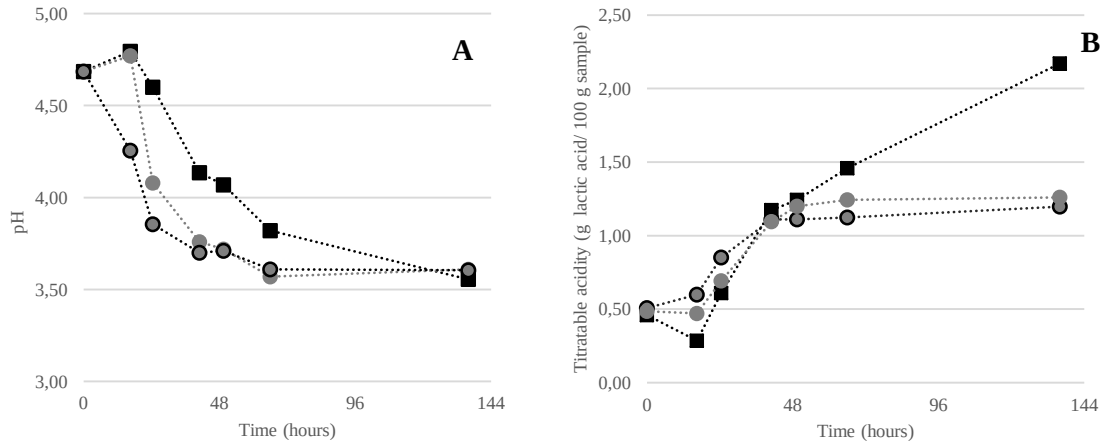

Figure 12 - pH (A) and titratable acidity (B) results of first backslopping from fermentations using pure starter cultures. Microflora is represented by (■), 1% backslopping by (●) and 10% backslopping by (◉). Points represent average and error bars the standard deviation.

As it can be observed on the previous figure, fermentations where backslopping was used were faster than the one that just used pepper microflora and they reached easily the desirable pH, 3.5, on nearly 70 h. As well as data from the first fermentation at 29 °C (**Figure 6A**) and data from fermentation using commercial starter cultures (**Figure 10A**)

both backsloppings took the same time to reach the desired pH, nearly 70 h. Therefore, it can be concluded that no improvements were reached with this first backslopping. Regarding acidity results, backsloppings were not so efficient since nearly 144 hours of fermentation acidity still remained on 1.25 % which is lower comparing with results of the previous fermentations referred above in **Figure 6B** and **10B,** nearly 1.75 %. It can be concluded that in terms of quickness of fermentation, one backslopping of a previous fermentation using pure cultures is as efficient as the use of mixed cultures for the first time.

LAB of first backslopping were quantified and results are presented in **Figure 13**.

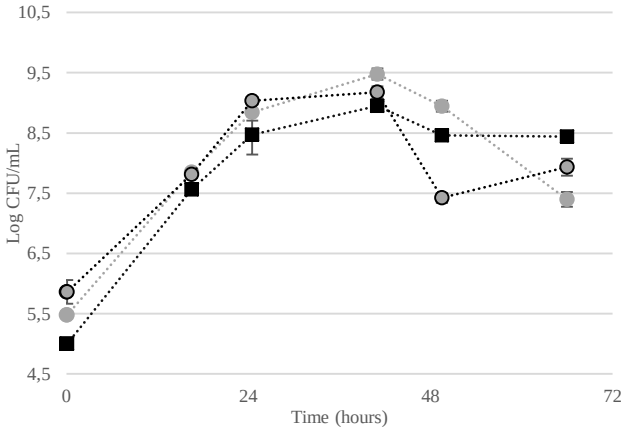

Figure 13 – Total viable LAB quantification results of first backslopping from fermentations using pure starter cultures. Microflora is represented by (■), 1% backslopping by (●) and 10% backslopping by (●). Points represent average and error bars the standard deviation.

Total viable LAB quantification is in agreement with what was said before about all fermentations with an initial inoculation since fermentations in which was used backslopping have more total viable LAB and fermentation reached the pH of 3.5 in less time. Initially, backslopping of 10% have more LAB, which is normal since more bacteria were inserted in the new fermentation. At the final stages, microflora remained with the highest number of LAB and bacteria from the fermentation that used 1% backslopping decreased, as well as bacteria from the 10% backslopping. This fact may be explained by the normal succession of LAB on lactic acid fermentations. The major part of LAB would probably be heterofermentative bacteria, characteristic of the first stages of these fermentations. Therefore, they were not adapted at lower values of pH and their number decreased with the time of fermentation due to the normal competition of bacteria in the

medium (Lahtinen et al. 2012). Contrarily, fermentation that just used microflora, reached higher acidity levels and remained at the final stages of fermentation because the normal succession may occurred.

In order to understand the results obtained with the first backslopping, a second one was performed. For this backslopping, LAB were quantified from the respective fermentation immediately before the inoculation. Second backslopping started with 1% of the previous one and total viable LAB were $2{,}50 \times 10^7$ cell/mL. The same happened for the second backslopping using 10% with $8{,}58 \times 10^7$ cell/mL of total viable LAB. Results of second backslopping are presented in **Figure 14**.

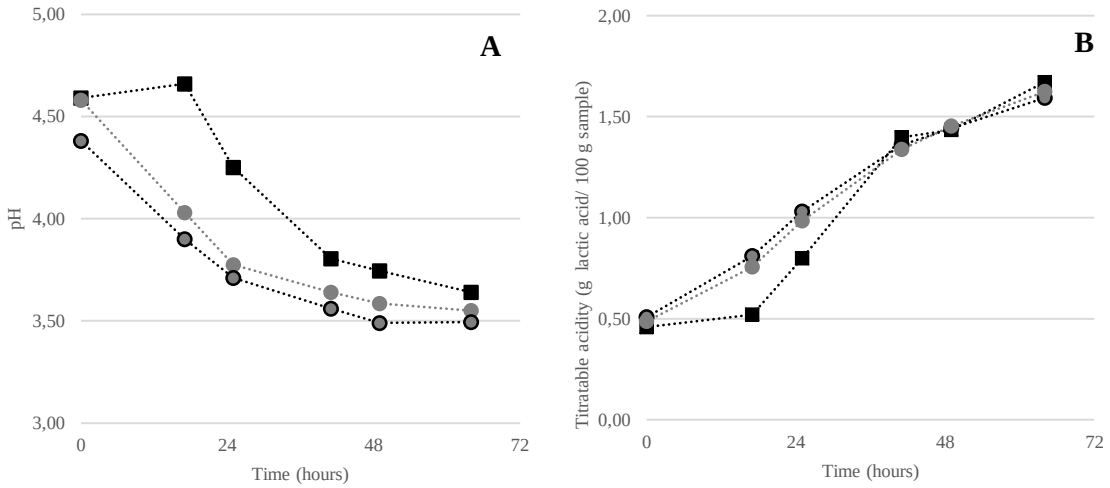

Figure 14 pH (A) and titratable acidity (B) results of second backslopping from the previous one. Microflora is represented by (■), 1% backslopping by (●) and 10% backslopping by (◘). Points represent average and error bars the standard deviation.

On the second backslopping, pH reached the desired value, 3.5, in 48h, the best time obtained since the beginning of this study. Acidity reached good values too, near 1.5 %. Comparing these results with the previous of first backslopping presented in **Figure 12**, it is possible to conclude that there were improvements on the efficiency of fermentation and the desired values were obtained in less time.

LAB were also quantified and the results are presented in **Figure 15.** This experiment showed that fermentation using 10% of backslopping started to have less bacteria at the final stages of fermentation. Therefore, it was decided to stop with the 10% of backslopping. Fermentation with 1% of backslopping had similar results with the

previous at the same conditions presented in **Figure 13.** However, in the present case, LAB did not decrease at the final stages of fermentation. Thus, a third fermentation was evaluated with this percentage of backslopping to study the viability of this process with the increase of the number of backsloppings.

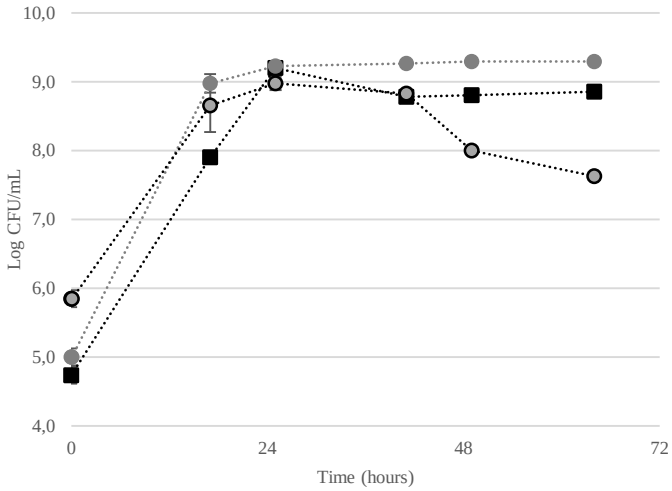

Figure 15 - Total viable LAB quantification results of second backslopping from the previous one Microflora is represented by (■), 1% backslopping by (●) and 10% backslopping by (◘). Points represent average and error bars the standard deviation.

In order to understand the viability of backslopping, a comparison between the three backsloppings of 1% was made and results of pH and acidity are in **Figure 16.**

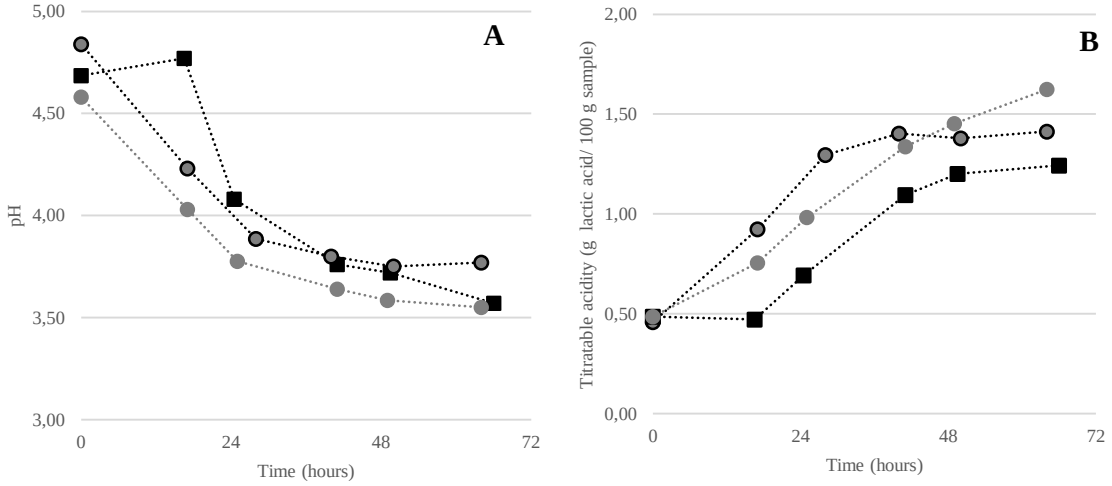

Figure 16 - pH (A) and titratable acidity (B) results of all fermentations where 1% backslopping was used from fermentations using pure starter cultures. First backslopping is represented by (■), the second backslopping by (●) and the third by (◘). Points represent average and error bars the standard deviation.

The results of **Figures 16A** and **16B** show that second backslopping was the more efficient in obtaining the lowest pH and highest acidity level. However, more studies on this subject are needed to understand the viability of this process especially due to the characteristic succession of bacteria on lactic acid fermentation.

On the same way, a comparison was made on total viable LAB quantification and results are present in **Figure 17**.

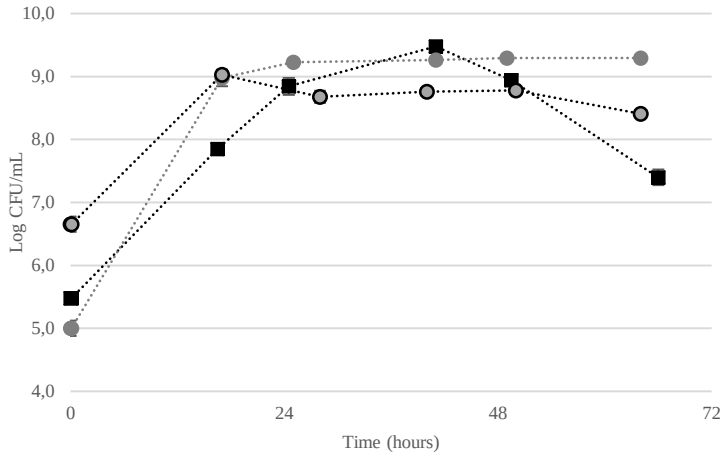

Figure 17 - Total viable LAB quantification results of all fermentations where 1% backslopping was used from fermentations using pure starter cultures. First backslopping is represented by (■), the second backslopping by (●) and the third by (◘). Points represent average and error bars the standard deviation.

As happened with pH and acidity levels, second backslopping was also the more efficient on the maintenance of LAB since these bacteria remained constant and at high levels until the final stages of the fermentation. Besides that, according to the data from the first backslopping, the maintenance of LAB concentration could not influence the success of the following fermentations since the decrease on bacteria of the first backslopping did not influence the success of the second. Thereby, the concentration of bacteria at the final stages of fermentation may not influence the success of the followings since it did not decrease below the recommended level to start a new fermentation which is 10^6-10^7 cell/mL and there is always the natural microflora of pepper to enrich the medium.

In order to further optimize the process, a backslopping of fermentations just using microflora was done. Backslopping was performed two times using microflora from the previous fermentations since these fermentations are capable of reaching the same pH and acidity levels of fermentations where starter cultures where introduced but they took much time.

Backslopping became the fermentations with starters faster, so the same process was tried utilizing just microflora and results are presented in **Figure 18**.

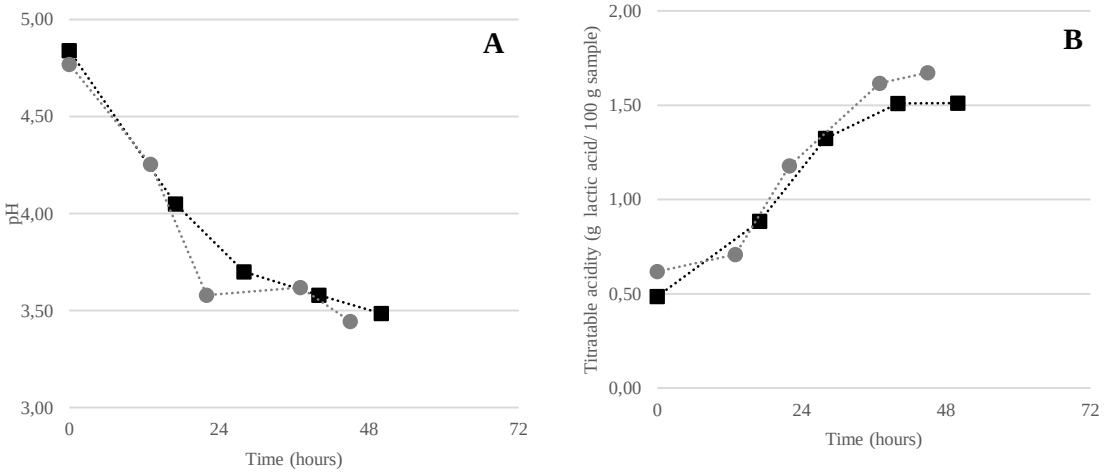

Figure 18 - pH (A) and titratable acidity (B) results of first and second backsloppings from 1% of fermentations using microflora. First backslopping is represented by (■), and second by (●). Points represent average and error bars the standard deviation.

Results show that the second backslopping was more efficient since it reached a pH near 3.5 in nearly 36h and high levels of acidity comparing with the first one. Evaluating the total viable LAB (**Figure 19**) on the second backslopping there was a decrease on the number of microorganisms however it remained above 8 log CFU/mL which still continue to be a high number to continue the backslopping process.

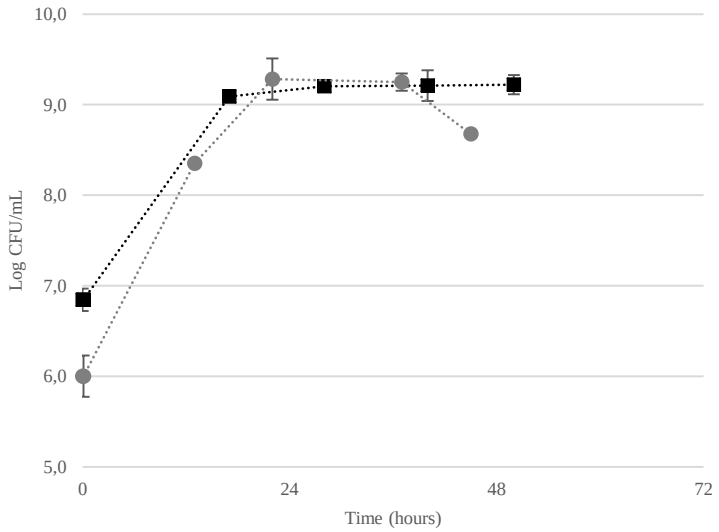

Figure 19 - Total viable LAB quantification results of first and second backsloppings from 1% of fermentations using microflora. First backslopping is represented by (■), and second by (●). Points represent average and error bars the standard deviation.

Comparing with the previous backsloppings where pure starter cultures were applied, these backsloppings using just the microflora reached lower values of pH and acidity in less time and total viable LAB were similar, between 8.5 and 9 log CFU/mL. Microflora seemed to be the best for backslopping process since it uses the microorganisms of pepper and fermentation reaches the desired values faster than the ones using pure starter cultures. However, it is important to note that further studies should be developed in order to understand deeply backslopping process on lactic acid fermentation and its viability as an improvement on the industrial process that already exists.

5. Microflora experiments

All experiments were evaluated at 29 °C and in spite of all the parameters that were studied, there was always a comparison with the normal development of microflora of red pepper which was utilized as a control. Therefore, there were several fermentations using different microflora with the same conditions. Comparing all these data, a significant difference was observed ($p < 0,05$). So, fermentations were significantly different. This result showed that, in spite of peppers came from the same region and belong to the same variety, microflora strongly influences the normal development of fermentation and this factor is difficult to standardize as it can be seen in the different figures (6, 8, 10, 12 and 14) where microflora reaches different values of pH and acidity.

6. Growth curves

Initially, shredded red peppers were heated at 60 °C, 70 °C and 90 °C in order to kill all the microflora and study the development of the pure starter cultures applied in this work, *Leuconostoc mesenteroides* and *Lactobacillus plantarum,* separately. Total viable LAB were evaluated and no microorganisms were found in dilutions 10^{-1} until 10^{-4}. Therefore, a temperature of 70 °C was chosen and pH and acidity results are presented in **Figure 20**.

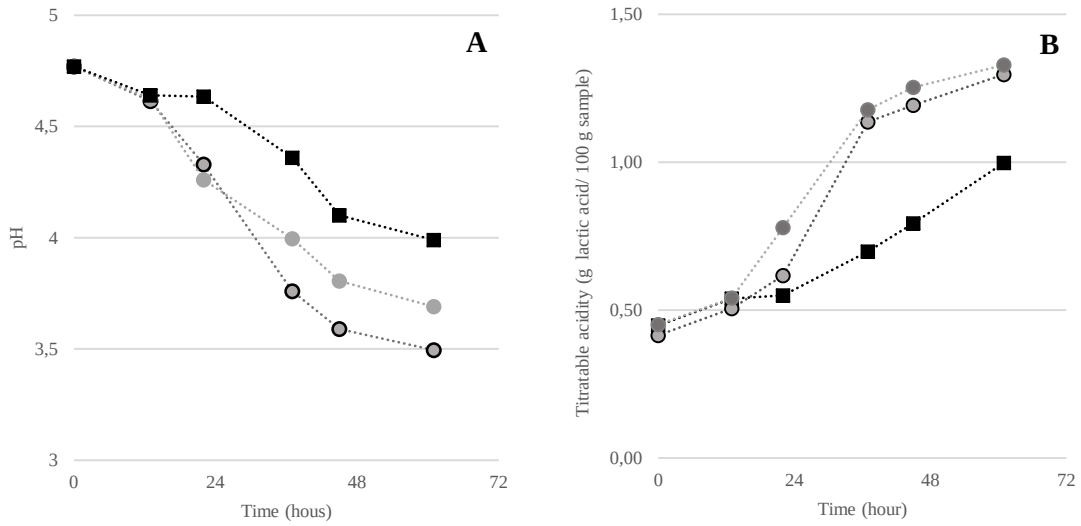

Figure 20 - pH (A) and titratable acidity (B) results of fermentations inoculated with *L. mesenteroides* (●), *L. plantarum* (◘) and without inoculation (■). Points represent average and error bars the standard deviation.

Fermentations where no starter cultures were inoculated had a decrease in pH and an increase in acidity which compromise the results since a sterilized environment was not obtained. Nevertheless, fermentation inoculated with *Lactobacillus plantarum* reached lower pH values than the fermentation inoculated with *Leuconostoc mesenteroides*. However similar values of acidity were obtained. Quantification of total viable LAB are present in **Figure 21**.

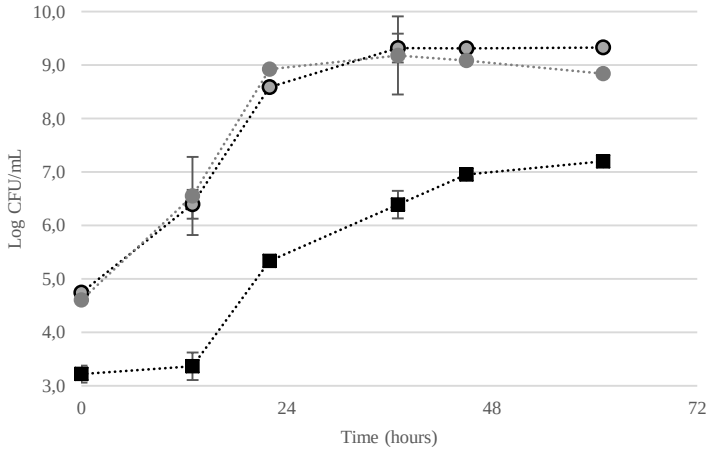

Figure 21 - Total viable LAB quantification results of fermentations inoculated with *L. mesenteroides* (●), *L. plantarum* (◘) and without inoculation (■). Points represent average and error bars the standard deviation.

Similarly, LAB from the inoculated fermentations were higher than the one without inoculation. Furthermore, fermentation with *Leuconostoc mesenteroides* started to have more microorganisms than the fermentation with *Lactobacillus plantarum* but, at the final stages, the contrary happened. This is what is in agreement with literature about the succession of hetero and homofermentative LAB (Lahtinen et al. 2012). Growing rates were calculated and they are presented in **Table 6**.

Table 6 – Growing rates of *Leuconostoc mesenteroides* and *Lactobacillus plantarum*. Control was not inoculated.

L. mesenteroides	0,20 h^{-1}
L. plantarum	0,18 h^{-1}
Control	0,12 h^{-1}

In spite of not being sure that the growing rates are only due to the bacteria inoculated, it can be concluded that at least the inoculation strongly influenced the fermentation since the results are in agreement with their natural development on lactic acid fermentations. Growing rate of *L. mesenteroides* was higher than the growing rate of *L. plantarum* since the first microorganism develops on initial stages of fermentation and the second on the final stages. Therefore, *L. plantarum* had more difficult to grow at the first stages of fermentation due to the initial unfavorable conditions. Nevertheless, the results are all very similar and their difference may not represent what really happens since in total viable LAB quantification, the behavior of both bacteria should be highly different as it is described in the literature by Stamer et al. (1971). In this study, *L. mesenteroids* started to decrease its viable number to 3 log CFU/mL after two days of fermentation and below a pH of 4 and *L. plantarum* remained with a viable number of LAB higher than 6 log CFU/mL with pH values lower than 4 and until 16 days of fermentation.

7. Sensory analysis

Sensory analysis compared the best results of this experiment and the target which is the red pepper paste that is used nowadays by Mendes Gonçalves. Thus, evaluated red pepper pastes were the final of backslopping using microflora and starter cultures which

are the second backslopping of fermentation using microflora and third backslopping of fermentation using starter cultures.

Initially, a discriminative test (triangle test) was done to realize the sensory differences between the two results of this work and the target. The results obtained are presented in **Table 7**.

Table 7 – Results of triangle test. M represents the second backslopping of fermentation using microflora, S represents the third backslopping of fermentation using starter cultures and T represents the target.

Triangle test 1		**Triangle test 2**	
Samples	Number of votes	Samples	Number of votes
A (T)	1	D (S)	4
B (M)	4	E (M)	0
C (T)	1	F (M)	3
No differences	3	No differences	2

To evaluate the number of correct answers from which it can be concluded that there is a difference between the samples, a statistical analysis was done according to Noronha (2003). Considering a binomial distribution and a level of significance of 5% with nine answers, the minimum of correct answers to consider that there is a difference between the values is six. Therefore, on both of the triangle tests, the number of correct answers were four. So, it was considered that no differences between the samples were identified. Red pepper pastes that were prepared with the fermented red pepper studied on this work were similar with the target.

After this test, just to understand the preferences of volunteers who realize the different on the previous test, an affective test was proposed and on triangle test 1, all volunteers preferred the sample M comparing with the T. This was an interesting outcome as M is the sample prepared with fermented red pepper prepared on this work which had the best results of all experiments. However, on triangle test where it was compared samples S and M, three of the volunteers who realize the differences preferred S sample. From this final affective test the preferences of people were S, then M and finally T and the principal reason was the red pepper flavor that the red pepper pastes prepared in this work had comparing with the target.

The form used on this sensory analysis is presented in **Annex A.3**.

CONCLUDING REMARKS AND FUTURE WORK

1. Conclusion

Vegetable fermentation represents the easiest and the most suitable way to increase the consumption of fresh-like vegetables. Lactic acid fermentation constitutes a safe method to transform vegetables into a valuable food. Particularly, red pepper paste constitutes a valuable food and a tradition on the preservation of pepper in Portugal.

Literature data showed that the combination of an ancient tradition with the present biotechnology knowledge allow the development of controlled vegetable fermentation processes where all the variables can be monitored. Temperature is one of them and with this work, as no special flavours are needed for the final red pepper paste, it was concluded that a temperature near 29 °C was the best one since pH and acidity desired values were reached in less time. Salt content was maintained ate 2% since is the lower value that favour the occurrence of lactic acid fermentations.

Additionally, one of the methods to have a controlled and safe process is the use of starter cultures which represents a way to control the natural microflora of the vegetables, most of the times unknown and difficult to control when there are different product suppliers. On the present work, diverse fermentations carried out at 29 °C using microflora demonstrated this diversification since different levels of pH, acidity and total viable LAB were obtained. Starter cultures were also evaluated at the present work. Mixed starter cultures of *L. mesenteroids* and *L. plantarum* as well as commercial starter culture, were capable of decreasing pH until 3.5 and increase acidity until 1.5% in less time (72 h) comparing with microflora of red pepper (144h).

On the other hand, an optimization of fermentation can be done through backslopping which represents a cheaper and reliable preservation method, when comparing with the use of starter cultures. Backslopping was carried out with pure starter cultures three times with 1 and 10% of previous serum, however, the best result was obtained for the use of microflora on the second backslopping which produced a fermented red pepper on nearly 36 hours. Regarding a sensory analysis that was performed, there was no significant differences between this last fermentation that took 36 hours and the company target (red pepper paste actually produced).

Vegetable fermentation is a very common method of preserving vegetables in some world regions but, due to the beneficial constituents and taste of fermented

vegetables, it should be widespread and well-studied, contributing to the diversification of food.

2. Future work

A solution for the improvement of red pepper paste production was studied and developed. However, there are several important aspects that should be studied in order to verify the application of this solution to the industrial processes.

First of all, HPLC results are important to see the products present on fermentations. To understand the evolution of a lactic acid fermentation, the decrease of pH and the increase of acidity are not enough. Thus, with HPLC results about lactic acid and acetic acid quantification it would be possible to know a little more about what kind of products microorganisms produced and its concentration during all the fermentation. This is important to understand what kind of reactions and microorganisms would be present on the different stages of fermentations and correlate its values with pH and acidity results.

Accurate information on the microbial genetic would be also an important topic to study since lactic acid fermentation have different acidic phases in which microorganisms have different behaviors. There are usually a succession in species depending on the acidification of the medium. Therefore, information about microorganisms and their role in fermentation would be interesting to study not only to know which species are present on the pepper microflora and know their development during fermentation but also to study the viability of backslopping.

The present work suggested backslopping as an alternative to produce red pepper paste in an efficient and fast way, however, more studies should be carried out to understand the viability of this process in terms of succession in microorganisms that are going to be introduced in a new fermentation and the number of times that they can be applied. Testing the best moment to take the bacteria may be a critical point that should also be studied in order to improve the efficiency of backslopping.

Experiments at industrial scale should also be done to understand the truly application of the present results. Process duration and final product characteristics are the principal advantages of backslopping and the ones that should be investigated within an industrial scale.

The final organoleptic properties of the product are a critical point since the sensory evaluation of the present work revealed that red pepper pastes with a strong flavor

of fresh pepper had an advantage. Therefore, the study of the development of the flavors with time should also be an important topic to evaluate and correlate with the raw material and the process involved in fermentation. Furthermore, more studies should be done to discover the influence of the addition of spices and other unusual vegetables to the fermentation medium since the principal effect of all spices is the inhibitory effect against yeasts and molds which are the principal problem in vegetable fermentation.

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ANNEXES

A.1 Calibration curves for HPLC UV/Vis

Calibration curves for lactic acid and acetic acid are presented on **Figure A1** and **Figure A2**, respectively, and were adapted from Meireles 2012.

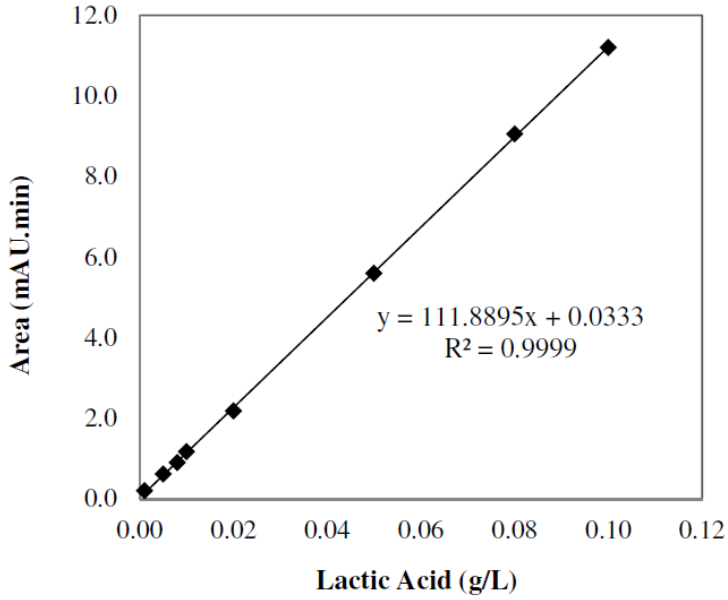

Figure A1 – Calibration curve for lactic acid.

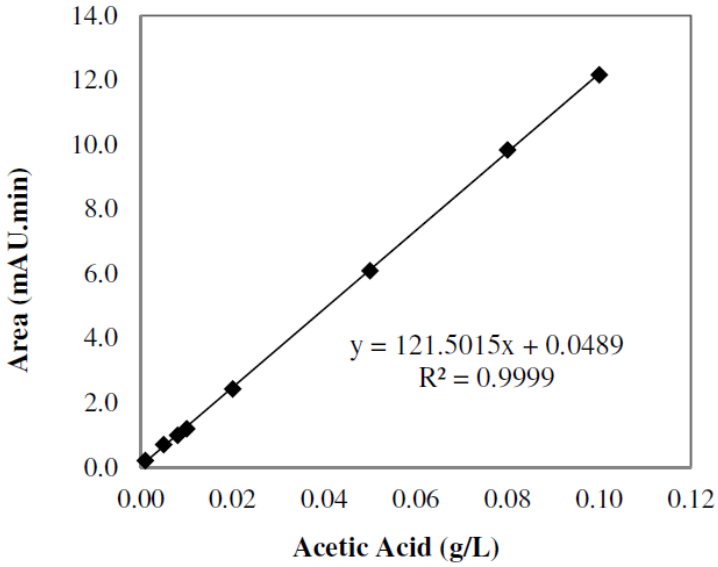

Figure A2 – Calibration curve for acetic acid.

A.2 HPLC results

HPLC was performed and results of lactic acid and acetic acid standards are present of the following **Figure A3** and **Figure A4**.

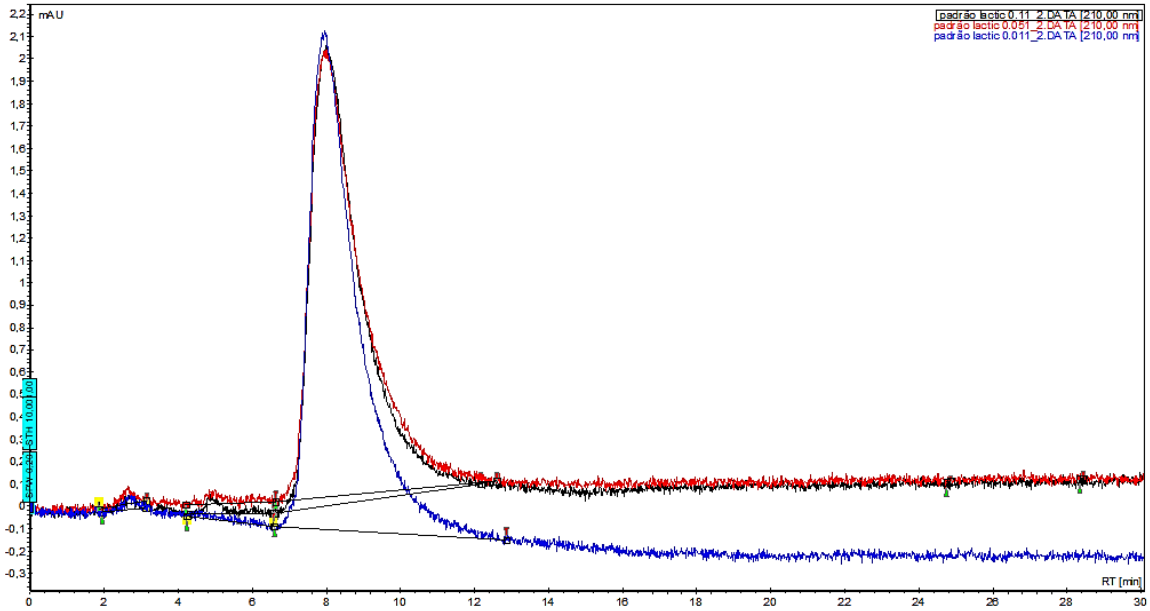

Figure A3 – Results of HPLC analysis of lactic acid standards.

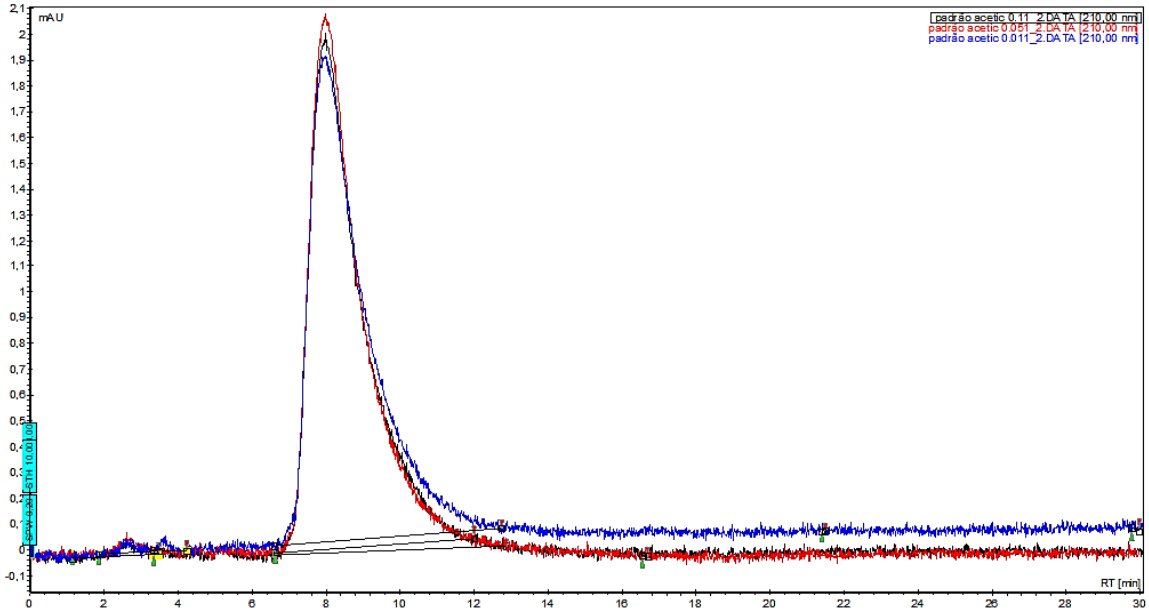

Figure A4 - Results of HPLC analysis of acetic acid standards.

As it can be seen, no differences were identified between the standard samples with different concentrations. A problem on HPLC was detected and the equipment was sent to the supplier.

A.3 Sensory analysis form

Prova Sensorial Massas de Pimento

1 – Triangular

M e target (A/B/C)

S e M (D/E/F)

Identificação amostras	Nº votos	Identificação amostras	Nº votos
A ( )		D ( )	
B ( )		E ( )	
C ( )		F ( )	
Sem diferenças		Sem diferenças	

2 - Afetivo

M e target (1/2)

S e M (3/4)

Identificação amostras	Nº votos	Identificação amostras	Nº votos
1 (M)		3 (S)	
2 (T)		4 (M)	