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Lactic acid fermentation of peppers: isolation, characterization and evaluation of starter cultures

Tatiana Tremoceiro Cordeiro

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Supervisors: Prof. Rogério Paulo de Andrade Tenreiro

Prof. Nuno Gonçalo Pereira Mira

Examination Committee

Chairperson: Prof. Jorge Humberto Gomes Leitão

Supervisor: Prof. Rogério Paulo de Andrade Tenreiro

Member of the Committee: Prof.ª Ana Cristina Anjinho Madeira Viegas

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ABSTRACT

Strains of *Lactobacillus plantarum*, *Lactobacillus brevis*, *Leuconostoc citreum*, *Lactococcus lactis*, *Weissella cibaria*, *Lactobacillus hilgardii* and *Enterococcus* spp. from spontaneous fermentations (0 and 3% NaCl) of different peppers were characterized by PCR fingerprinting using M13 and $(GTG)_5$ primers and identified by partial 16S rRNA gene sequence. Strains of *Lb. plantarum*, *Lb. brevis*, *Lc. lactis* and *Leuc. citreum* were selected to be used as starter cultures for controlled fermentations of bell and hot peppers, based on their kinetics of growth, acidifying ability and production of lactic acid.

With the main goal of producing innovative products with distinct organoleptic and nutritional qualities, different pepper fermentations were assayed aerobically at 28ºC. Hot and bell peppers pastes (0 and 5% of NaCl) were used and the selected strains were inoculated. Uninoculated pastes in same conditions were used as control. Cell numbers of starter cultures (9 log CFU mL^{-1}) were 1000 times higher than hypothetic lactic acid bacteria present in vegetables. The pH reduction and lactic acid production was higher in pastes with *Lb. plantarum*, while the sugar consumption was higher with *Lb. brevis*.

PCR fingerprinting showed that *Lb. plantarum* strain was the best one persisting over fermentations, although *Lb. brevis* and *Leuc. citreum* strains also persisted in bell peppers pastes (0% and 5% of NaCl, respectively).

Twelve days after fermentation, sensorial analysis showed that the pastes with lactobacilli have the best organoleptic properties. These assays revealed an association of quality of pepper pastes with lactic acid production and final fermentation pH.

Keywords: Fermentation; Pepper paste; Lactic acid bacteria; Starter cultures; *Capsicum annum.*

RESUMO

Estirpes de *Lactobacillus plantarum*, *Lactobacillus brevis*, *Leuconostoc citreum*, *Lactococcus lactis*, *Weissella cibaria*, *Lactobacillus hilgardii* e *Enterococcus* spp., obtidas por fermentações espontâneas de pimentos (0 e 3% NaCl) foram caracterizadas por PCR *fingerprinting* (primers M13 e $(GTG)_5$) e identificadas por sequenciação parcial do gene 16S rRNA. Baseado no crescimento, capacidade de acidificação e produção de ácido-láctico, estirpes de *Lb. plantarum*, *Lb. brevis*, *Lc. lactis* e *Leuc. citreum* foram selecionadas como culturas de arranque em fermentações de pimentos.

Com o objetivo de produzir produtos inovadores com distintas qualidades organoléticas e nutricionais, foram realizadas fermentações de massas de pimentos picantes e doces (0 e 5% NaCl) a 28ºC aerobicamente, utilizando como inóculo as estirpes selecionadas. Massas não inoculadas foram usadas como controlo, nas mesmas condições. O número de células de culturas de arranque (9 log CFU mL^{-1}) foi 1000 vezes superior ao número de bactérias lácticas presumíveis em vegetais. A redução de pH e a produção de ácido láctico foi maior em massas com *Lb. plantarum*, enquanto o consumo de açúcar foi maior com *Lb. brevis*.

Baseado no PCR *fingerprinting*, a estirpe de *Lb. plantarum* foi a melhor, uma vez que persistiu durante as fermentações. No entanto estirpes de *Lb. brevis* e *Leuc. citreum* também persistiram em massas de pimento doce (0% e 5% NaCl, respetivamente).

Doze dias após fermentação, a análise sensorial indicou que massas com lactobacilli apresentam melhores propriedades organoléticas, revelando uma associação entre a qualidade das massas e o ácido e o pH produzido no final da fermentação.

Palavras-chave: Fermentação; Massas de pimento; Bactérias lácticas; Culturas de arranque; *Capsicum annum.*

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
CFU	Colony-forming unit
CoA	Coenzyme A
CYC	Cycloheximide
D-GPT	D-glutamate-pyruvate transaminase
EDTA	Ethylene diamine tetra acetic acid
EMP	Embden-Meyerhorff-Parnas pathway
G + C	Guanine plus cytosine
G6P-DH	Glucose-6-phosphate dehydrogenase
GRAS	Generally Recognized As Safe
LAB	Lactic acid bacteria
LDH	Lactate dehydrogenase
MRS	Mann Rogosa and Sharpe (Agar)
NADH	Nicotinamide adenine dinucleotide + hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate + hydrogen
NAUC	Net area under curve
NCBI	National Center for Biotechnology Information
PC	Principal Component
PCA	Principal Component analysis
PCR	Polymerase Chain Reaction
PGI	Phosphoglucose isomerase
SDS	Sodium Dodecyl Sulfate
TE	Tris-EDTA
YEPGA	Yeast extract peptone glucose agar

1. INTRODUCTION

1.1. Fermentation

The fermentation term is derived from Latin verb "*fervere*" (to boil) and it was defined in 1857 by Louis Pasteur, microbiology father's, as "La vie sans l'air" or life without air. The denomination of term "to boil" comes from the production of carbon dioxide bubbles formed from available sugars [Stanbury *et al.*, 2013].

The fermentative process is considered a preservative method in a food processing and one of the oldest methods of bio-preservation. It depends on the activity of microorganisms in the production of different metabolites, leading to an inhibition of spoilage microbiota in food [Ross *et al.*, 2002].

Fermentation is an anaerobic process, supported essentially by obligated anaerobic organisms in an anoxic environment. However there are other types of microorganisms (facultative aerobes) characterized by surviving in settings with or without oxygen, which can also carry fermentative processes [Mehta *et al.*, 2012]. This is characterized as a metabolic procedure in which carbohydrates are oxidized, generating energy without any external oxidizing agent [Jay, 1992].

The catabolism of sugars results in the production of reduced pyridine nucleotides (NADH) and under anaerobic conditions occurs the oxidation of NADH through the reduction of an organic compound (pyruvate) obtained from catabolic pathway (glycolysis) [Stanbury *et al.*, 2013].

Many types of fermentation processes can occurs depending on the end products obtained from pyruvate. Thus, NADH is regenerated by the reduction of pyruvate to lactic acid, ethanol or acetate depending on the intended fermentation [Mehta *et al.*, 2012].

Lactic acid fermentation is one of the most practical and widely applied methods for preserving and enhancing organoleptic and nutritional quality of food [Tamang *et al.*, 2005]. The main end product of this process is lactic acid, which is responsible for taste, microbiological stability and safety of food [Das and Goyal, 2012].

Lactic acid (2-hydroxypropionic acid) results from the oxidation of glucose and was isolated for the first time in 1970 from sour milk. This compound is water soluble, non-volatile, acidulant and it can occur in several food products, such as dairy products, fermented fruits, vegetables and sausages [Benninga, 1990].

Lactic acid can occur in different isomeric forms, L-lactic acid, D-lactic acid or as its racemic mixture, depending on the NAD^+-dependent lactate dehydrogenases (LDH) present in each strain, which differ in their stereospecificity producing the form of L(+) or D(-) lactic acid [Axelsson, 2004]. Biologically, the L- isomer is more important than the D-isomer, since this last one is not metabolized by humans, being eliminated by kidneys [Montet *et al.*, 2014].

Based on dissimilation of glucose, the lactic acid bacteria can assume two types of fermentations, homolactic and heterolactic, which use a glycolytic or phosphoketolase pathway respectively. Both fermentations generate ATP by substrate-level phosphorylation [Endo and Dicks, 2014].

1.2. Lactic acid fermentation of vegetables

Lactic acid fermentation has been used in food for centuries, but in the last few years it has received special attention for becoming the main bio-preservation method, improving safety and shelf-life, mostly in vegetables. Although vegetables have low sugar content, they are very rich in minerals and vitamins which provides a natural environment for lactic acid bacteria [Montet *et al.*, 2014].

The lactic acid fermentation is a low cost process, so it is much used in biotechnological processes, promoting the organoleptic quality, improving the palatability, digestibility and nutritional value of food. This fermentation also promotes the prolongation and preservation of shelf-life through formation of organic acids (lactic acid, acetic acid, formic acid, propionic acid), ethanol and bacteriocins, produced by bacteria responsible for this process. These act as inhibitory metabolites, inhibiting pathogens or removing toxic compounds [Di Cagno *et al*, 2013].

Some environmental factors can affect the fermentative process of vegetables, namely the quality of raw material, chemical composition, concentration of salt, temperature, the original microbiota and harvesting conditions. Thus, depending on these factors, the quality of products is variable [Andersson *et al.*, 1988; Di Cagno *et al*, 2013].

Peppers Fermentation. Peppers are one kind of vegetables used in lactic acid fermentation. These are annual herbaceous plants, members of Solanaceae family, belonging to *Capsicum annum* [Di Cagno *et al.*, 2013] or *Capsicum chinense* (less known) species.

Capsicum genus includes 5 main species and more than 200 varieties, which can vary in shape, size, flavour and sensory properties. The bell peppers (sweet) and chillies (hot), which were used in this thesis, are examples of different varieties of *C. annum* species.

The varieties of *C. annum* are differentiated essentially in colour, which can be altered during ripening, from green to orange or red, presenting different compounds that define the colour of each one (chlorophylls or carotenoids, respectively). The type of carotenoids can be variable, as such the concentration of these, leading to different economic values [Peréz-Gálvez, *et al.*, 2006].

Peppers are cultivated in regions of temperate and warm climate and present various characteristics such as aroma, colour and pungency, which increase the consuming of this vegetable. Due to their antioxidant activity, a diet in pepper could be very rich, leading to the prevention of several diseases, as cancer, cardiovascular diseases and obesity [Di Cagno *et al.*, 2009; Asnin and Park, 2015].

The way to consume this type of vegetable can be variable, offering a big diversity around these products. So, these can be consumed from mature or immature, raw or in conserves (pickles), to pastes

and dehydrated products [Di Cagno *et al.*, 2009]. Recently, peppers have been widely used in the production of pastes, which can have hot or sweet tasteful characteristics [Alberto et al., 2013].

Production of pepper paste. Pepper paste is a traditional fermented product obtained from *Capsicum annum* species that include different types of peppers (sweet or hot). This shows benefits at level of health, being consumed in many countries, as Turkey, China, Mexico and Korea. The production of this fermented food at industrial level depends of several factors, such as initial microflora, thermal processes, pH, total soluble solids, addition of salt and chemical preservatives [Kuleaşan and Okur, 2012].

The organoleptic characteristics pretended in this fermented product, like aroma, taste and colour can be obtained through different processes such as cooking, spontaneous fermentation conducted by the microorganisms present in raw material or solar drying processes. In cooking processes different temperatures can be used, nevertheless inadequate temperatures can promote problems relating with the colour of pepper pastes. However these processes are useful in the inhibition of spoilage microorganisms, since with higher temperatures occurs the destruction of pectin, degrading enzymes, leading to a higher viscosity of pepper pastes [Kuleaşan and Okur, 2012].

To avoid the use of thermal treatments, the sanitation according to general health department to wash vegetables and fruits is a possibility. Although in these cases the microbiota present in raw material is not completely removed, the characteristics of peppers stay intact. Thus, other parameters can be taken into account such as water activity and concentration of salt. The last one can be used in different concentrations, although it should be used lower than 10%, but there is little information available on fermentation of peppers [Bozkurt and Erkmen, 2004].

The procedure to prepare pepper pastes, sweet or hot, is similar in different experiments mentioned in literature. The peppers are washed and broken, the seeds are removed and then occurs the grind of peppers. After this, techniques with thermal processes can be applied and salt can be added to pepper paste [Kuleaşan and Okur, 2012].

In hot pepper paste, two techniques can be used to concentrate the peppers, reducing the level of water in peppers and avoiding contaminants. These concentration techniques can occur in an open pan or under vacuum, where the evaporation occurs through the natural conditions with climate elements or with vapour processes, respectively [Bozkurt and Erkmen, 2014].

At the present time, the industrial process of this fermented product is more important, since the traditional method does not respond to required necessities [Kuleaşan and Okur, 2012].

1.3. Processes of lactic acid fermentation in vegetables

Lactic acid fermentation to the production of fermented vegetables can be based on spontaneous fermentation, with the development of the natural microbiota present in raw material, or can use a starter culture, where is added at least one microorganism to a raw material – controlled fermentation [Leroy and De Vuyst, 2004].

In both processes of lactic acid fermentations, preservation methods can be used to promote the best fermentation with present microorganisms. Thus, sometimes salt is used to help preservation of vegetables, although the fermentation can also occur in the absence of salt, being dependent only of other intrinsic conditions (raw material, temperature, pH, etc.).

1.3.1. Spontaneous fermentation

The traditional method to preserve vegetables is based on spontaneous fermentation that use the natural microbiota present on raw material. There are many consumed vegetables using fermentation as a preservation method, such as cabbages, carrots, beets, peppers, olives, cucumbers and each one harbours a dominant and constant microbiota [Sánchez *et al.*, 2000]. This spontaneous process results from the competitiveness of established microorganisms, where those best adapted dominate the process.

Spontaneous fermentation is a sensible process, as Lactic Acid Bacteria (LAB) vary with the quality of the raw material, temperature, harvesting conditions and other intrinsic characteristics, leading to variations in organoleptic characteristics of product [Gardner, *et al.*, 2001]. In addition to epiphytic microbiota, vegetables contain potential spoilage microbes. Thus, to avoid the deterioration of the product it is possible to enrich the plants environment so that the LAB have better conditions to grow, since they are the least prevalent microorganisms. Possible solutions are enriching with salt or by the addition of proteins [Montet *et al.*, 2014].

The growth of LAB is dependent on the chemical (subtracts, salt condition, pH) and physical factors (raw material, temperature), which vary over time, leading to a succession of bacteria and sometimes yeasts may be present. The natural microorganisms that conduct the spontaneous fermentation have an interest since they improve the health of fermenting foods [Di Cagno, *et al.*, 2013].

The bacteria responsible for spontaneous fermentation produce organic acid, such as lactic acid which decreases the pH of vegetables, with subsequent inhibition of the potential spoilers. However, in spontaneous fermentation the pH stabilizes only after 4 to 6 days of fermentation. To accelerate this process other types of fermentation with inoculation of starter cultures are used [Gardner, *et al.*, 2001 in Aukrust *et al.*,1994].

1.3.2. Controlled fermentation

A controlled fermentation is used if a standardized end product is required. Through this process it is possible to obtain a high degree of control over the fermentation process, that is very important in the industrialization [Montet *et al.*, 2014]. The controlled fermentation avoids the risk of fermentation failure, the inadequate inhibition of spoilage and pathogen microorganisms and undesirable organoleptic variations through the use of predefined and isolated cultures. For that reason the use of this process is increasing in vegetable fermentation [Di Cagno, *et al.*, 2013].

The spontaneous fermentation is a process a bit longer, leading to a competition among microorganisms for nutrients, to produce their metabolites, and the controlled fermentation with inoculated cultures may avoid this by shorten the initiation phase (lag phase) [Holzapfel, 2002]. To control the fermentation, the inoculated bacteria have to exclude and compete with the natural microbes, resulting in good sensory properties on end products [Gardner *et al.*, 2001].

Due to their nutritional, technological and probiotic ability, and also because they cause a rapid acidification of the raw material, the lactic acid bacteria have a central role in the fermentation process and so they are used on the control of lactic acid fermentation of vegetables [Florou-Paneri *et al.*, 2013]. Thus, it is usual the utilization of LAB starter cultures that enables making food products with standard quality in a shorter time and prevents economic losses due to spoilage [Tamminen *et al.*, 2004].

Starter culture. The starter cultures are characterized as preparations with living microorganisms, which are applied to a raw material with the intention to produce a fermented food, by accelerating and conducting its process [Leroy and De Vuyst, 2004].

Furthermore, the starter culture is also responsible for the control of the natural microbiota. Thus, these cultures have ensure the microbial safety, so they must be free of pathogens; they have technologic effectiveness, being dominant in relation to natural microbes; and they should offer one or more organoleptic, technological, nutritional or health advantages [Gardner *et al.*, 2001]. The use of starter cultures is important to guarantee not only hygiene and safety, but also to get products with consistent sensory and nutritional properties [Mozzi *et al.*, 2015].

The starter cultures can be divided into three classes: 1) “Undefined cultures”, based on the use of fermenting substrate, from a selected process with end products of good quality; 2) “Single/multi-strain cultures”, with one or more defined strains, respectively; and 3) “Back slopping”, based on inoculation of the raw material with a small quantity of a previously successful batch [Leroy and De Vuyst, 2004].

Sometimes, chemical food additives (nitrite, sulphite, propionic acid, and others) are added to better preserve food. To avoid the addition of these chemicals, the use of starter cultures may be helpful to prevent microbial spoilage, since they are responsible for producing natural antimicrobials [Leroy and De Vuyst, 2004].

Selection of a suitable starter culture. The use of starter cultures is important to standardize the fermentation by controlling the natural microbiota. Its selection is based predominantly on the competitiveness between the starter and the natural microbes and on the sensory properties of the resulting products. Besides, the starter cultures should also eliminate undesirable side effects, such as formation of D-lactic acid or a racemate of lactic acid, and the formation of biogenic amines [Leroy and De Vuyst, 2004].

The environmental adaptation, such as pH, presence of inhibitory compounds and concentration of fermentable carbohydrates, is very important in selection process, since it affects all the metabolic features. Another criterion for selection of starter cultures is the inability to synthesize of hydrogen peroxide (strong oxidizing agent) that can react with organic material, causing degradation of antioxidant components, and undesired loss of the product colour [Di Cagno *et al.*, 2013].

1.3.3. Salted Fermented Vegetables

The addition of salt to the vegetables can be done in both types of fermentations mentioned above, to enhance the quality of fermentation. It may be added in dry form or as brine solution depending on the plant material. The concentration of salt regulates the type of microorganisms and modulates their growth, affecting the quality and safety of the fermented raw material. The level of salt is variable, due to the fact that some lactic acid bacteria are limited to about 2.5 – 4.0% of salt and others are more resistant, tolerating up to 8% of NaCl, like *Lactobacillus plantarum* and *Lactobacillus brevis* [Fleming and McFeeters, 1981]. The maximum salt concentration tolerated by LAB in vegetables is around 10% in a fermentation, being the growth slower or inexistent with high concentrations of NaCl [Andersson *et al.*, 1988].

The salt is very important in lactic acid bacteria, since it promotes the plasmolysis of nutrients, which are subsequently used by LAB to assure their growth and maintenance, producing acid to inhibit spoilage. Thus, salt leads to the obtainment of products with desired sensory qualities, like flavour, texture and aroma; and reduces the effects of softening enzymes, using a low energy input [Andersson *et al.*, 1988].

1.4. Disadvantages of the fermented vegetables

Although fermentation of vegetables is largely used as a bio-preservative process, there are also some inconvenients associated to it.

Some lactic acid bacteria are able to produce biogenic amines during the process of food fermentation by amino acid decarboxylation, which can be harmful for human health. When ingested in high concentrations, the biogenic amines might induce some health problems as headaches, respiratory distress, heart palpitations and other diseases. One very well-known biogenic amine and very toxic compound is histamine which can be potentiated by other amines present [Lonvaud-Funel, 2001].

The biogenic amines are produced by amino acid decarboxylases present in some LAB, such as strains of *Leuconostoc* spp. (*Leuc.*), *Lactobacillus* spp. (*Lb.*) and *Pediococcus* spp. (*P.*), damaging food fermentation and the availability of free amino acids, which can occur in food or be liberated by proteins (proteolytic activity). In spontaneous fermentation, the probability of existing biogenic amines is higher because the microbiota is undefined [Buckenhüskes, 1993; Montet *et al.*, 2014].

1.5. Microbiota involved in fermented vegetables

Studies regarding microbiota in fermented vegetables were initiated in the early 1900s and include a large number of microorganisms. The population of microorganisms in vegetables is variable, being able to fluctuate between 5.0 and 7.0 log cfu g^{-1} and has an essential role in the biological fermentation process [Di Cagno *et al.* 2013]. These microorganisms found in vegetables can change depending on the physical and nutritional conditions of each raw material, but the normal microbiota of vegetables is constituted by yeasts, molds, Gram- and Gram+ bacteria, including in the last ones the group of lactic acid bacteria [Di Cagno *et al.*, 2013].

Lactic acid bacteria (LAB) are widespread microorganisms and they have been associated with food and feed fermentations, being considered bacteria with beneficial characteristics to health. These are responsible to keep or enhance the nutritional, safety, organoleptic and shelf-life properties of vegetables, through the conversion of sugars in organic acids (lactic acid fermentation). Since LAB present these characteristics, they are frequently inoculated in several products to produce aroma compounds and carbon dioxide [Rossetti and Giraffa, 2005]. On the other hand, some species of the *Pediococcus* genus can cause the deterioration of foods, leading to spoilage of food [Florou-Paneri *et al.*, 2013].

The LAB are only a small part of the microbiota present in vegetables, with 2.0 – 4.0 log cfu g^{-1} but there is a big diversity in this group. They can be found in several vegetables, including tomatoes, carrots, beans, cucumbers, peppers and others. The prevalent LAB species associated with fermentation of vegetables are *Leuconostoc mesenteroides*, *Lactobacillus brevis*, *Lactobacillus plantarum*, *Lactococcus lactis*, *Pediococcus pentosaceus*, *Weissella cibaria* and *Weissella confusa* (Figure 1) [Andersson *et al.*, 1988].

In vegetable fermentation there are several strains involved in various stages of this process, existing a succession of hetero- and homo- fermentative lactic acid bacteria. In the beginning of the fermentation, strains of *Leuconostoc mesenteroides*, *Lactobacillus brevis* or related species of LAB are present (heterolactic fermentation), since these grow more quickly than other LAB strains. Those strains lead to a quickly decrease of batch pH due to the production of acid, inhibiting spoilage, and thereafter LAB using the homofermentative pathway carry out the fermentation. Strains of *Lactobacillus plantarum* are usually responsible for this process, because they produce almost exclusively lactic acid in the last phase of fermentation with remaining sugars and they have ability to tolerate very low pH [Breidt *et al*, 2013].

	Lactic acid bacteria species													
Source	*Lb. plantarum*	*Lb. pentosus*	*Lb. fermentum*	*Lb. curvatus*	*Lb. brevis*	*Lb. paraplantarum*	*Leuc. mesenteroides*	*W. soli*	*W. confusa*	*W. cibaria*	*E. faecalis*	*E. faecium*	*P. pentosaceus*	*P. cerevisiae*
Tomatoes	•				•				•	•	•	•	•	
Marrows	•													
Carrots	•						•							
Cucumbers	•	•					•						•	
Eggplants	•	•	•		•		•							
Cabbages	•				•	•	•						•	
Peppers				•			•		•	•				
Kimchi	•				•		•							
Sauerkraut	•				•		•							
Pickles	•				•		•							•

Figure 1. Species of lactic acid bacteria isolated from vegetables. *Lb.*: *Lactobacillus*, *Leuc.*: *Leuconostoc*, *W.*: *Weissella*, *E.*: *Enterococcus*, *P.*: *Pediococcus*. Based on Di Cagno et. al. (2013) and Breidt et al. (2013).

1.6. Taxonomy of lactic acid bacteria

In 1873, the first pure culture of a lactic acid bacterium, designated by *Bacterium lactis* (*Lactococcus lactis)*, was obtained by J. Lister [König and Fröhlich, 2009]. Posteriorly, in 1919 comes up the term "lactic acid bacteria" as a group of microorganisms emerged by Orla-Jensen and it was used to refer to "milk-souring microorganisms". In the first monograph by Orla-Jensen, the lactic acid bacteria were described in relation to its cellular morphology, mode of glucose fermentation, capacity to grow in several temperatures and sugar utilization. Being so, only four genera were recognized with these criteria, namely *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* [Wright and Axelsson, 2011].

Phylogenetically, Gram positive bacteria are divided into two major branches, the bacterial phylum Firmicutes, with a DNA base composition of less than 55 mol % G + C (Guanine plus Cytosine), and the phylum Actinobacteria, which contains more than 55 mol % G + C. LAB are included in the phylum Firmicutes, being grouped in the Bacilli class of this phylum, which contains two orders, the Bacillales and the Lactobacillales, with 12 and 6 families, respectively. The LAB are found in this last order with 40 genera and six families that include Aerococcaceae (7 genera), Carnobacteriaceae (16 genera), Enterococcaceae (7 genera), Lactobacillaceae (3 genera), Leuconostocaceae (4 genera) and Streptococcaceae (3 genera) [Holzapfel and Wood, 2014].

Initially, classifications of LAB were exclusively based in phenotypic characteristics. Thus, based on growth at different temperatures and their fermentation of hexose and pentose sugars, lactobacilli were divided into three subgenera, “Thermobacterium” (obligately homofermentative), “Streptobacterium” (facultatively heterofermentative) and “Betabacterium” (obligate heterofermentative). However, with the emergence of molecular methods, it was possible to understand that the subdivision is not consistent and other subgroups were created. This way, the genus *Lactobacillus* could be divided into the obligatory homofermentatives (no fermentation of pentoses), obligatory heterofermentatives (hexoses and pentoses fermented and gas production from hexoses) and the facultative heterofermentatives (hexoses fermented by glycolysis and pentoses fermented by phosphogluconate pathway; gas produced from hexoses), this being the designation used nowadays [Klein *et al.*, 1998].

Besides phylogenetic, the natural diversity and the relationships between organisms have been based in several data like phenotypic and genomic, chemotaxonomic and metabolic characteristics, and must be applied in combination – polyphasic taxonomy. Several taxonomic methods are included in polyphasic approach [Felis and Dellaglio, 2007].

To understand the phylogenetic relationships in LAB, the comparative analysis of 16S rRNA gene sequence is the best approach, since this gene is present in all bacteria; is highly conserved; not subjected to horizontal gene transfer between organisms and has regions with different conservation that allow the reconstruction of phylogenies at several taxonomic level [Felis and Dellaglio, 2007]. However other genomic characterization approaches are also used, as ribotyping, polymerase chain reaction (PCR) fingerprinting analysis, and others.

Concerning phenotypic analysis it is performed the analysis of carbohydrate fermentation patterns, glucose fermentation products, the determination of isomers of lactic acid production, type of peptidoglycan structure of the cell wall and the DNA G + C content [Vandamme *et al.*, 2014]. Through these methods it was possible to obtain an evident characterization of the group of lactic acid bacteria.

1.7. Characterization of LAB

The Lactobacillales order constitutes a diverse group of Gram positive microorganisms, known by their high production of lactic acid as major or sole end product. It is difficult to define LAB as a single group, although these can be characterized as nonsporing, aero- and acid tolerant, non-motile, organotrophic and a strictly fermentative microorganisms [König and Fröhlich, 2009]. They can present various kinds of cells, from coccus to rods, being spherical or long, and with several forms, according to genus or species, such as, single cells, chains, pairs, groups or tetrads [De Vos *et al.*, 2009]. The cell division of lactic acid bacteria occurs essentially in one plane, except sometimes in some genera as *Pediococcus*, *Aerococcus* and *Tetragenoccus*, which can present tetrads [König and Fröhlich, 2009]. Furthermore, lactic acid bacteria are catalase and oxidase negative [König and Fröhlich, 2009].

As mentioned above these bacteria contain non-motile microorganisms but some species also can have peritrichous flagellation. This fact is related with the medium and the age of culture and it is sometimes observed only in isolation [De Vos *et al.*, 2009].

1.8. Ecology and habitat of LAB

Lactic acid bacteria are fermentative bacteria that occur naturally in nutrient-rich environments, where LAB have their own niches. They are capable to colonize a variety of habitats, since they have a high metabolic capacity, making them good acid food fermenters [Giraffa, 2014]. As energy providers and nutrients, they require, carbohydrates, amino acids, peptides, nucleic acids and vitamins because they don't have many biosynthetic capabilities, and for that reason they need habitats with a rich nutrition supply (high levels of solute carbohydrate, protein breakdown products, vitamins, and sometimes reduced oxygen tension) [Hammes and Hertel, 2014].

These bacteria are found in a large variety of environments, such as plant and animal raw materials and the fermented food products such as dairy, meat and fish, vegetable and fruits, sauerkraut, beverages, and they can also occur in cavities of human and animals. LAB are responsible for a healthy microbiota of the human gut and sometimes some species are used in food fermentation as starter cultures, as mentioned above [König and Fröhlich, 2009].

The LAB, with the production of lactic acid, are responsible for the decrease of pH of their substrates, contributing for their wide distribution and their successful establishment in several habitats, inhibiting the growth of other bacteria that are responsible for decomposition of material food and spoilage [Giraffa, 2014].

1.8.1. Metabolism and physiology

These bacteria use sugars, with substrate-level phosphorylation, to obtain carbon and energy sources for their growth in fermented raw material. Thus, LAB can use different metabolic pathways to produce ATP, which is required to transport solutes across the cell membrane and for biosynthesis.

These groups of bacteria are generally mesophilic, but some strains can also grow at low (5ºC) or high temperatures (45ºC). LAB grow in pH of approximately 6.0, but growth can also happen at 3.2 and 9.6, presenting a broad range of pH [Caplice and Fitzgerald, 1999].

Lactic acid bacteria are a group of bacteria that live in a threshold of anaerobic-to-aerobic life and for that reason are called aerotolerant bacteria [De Vos *et al.*, 2009]. Although most strains are aerotolerant, optimal growth is achieved under microaerophilic or anaerobic conditions [De Vos *et al.*, 2009]. Given that they don't present cytochromes and are unable to synthesize porphyrins, they do not carry out oxidative phosphorylation [Madigan *et al.*, 2011]. But under certain circumstances, catalase and even cytochromes may be formed, if heme (haemoglobin) is added to growth medium, resulting in respiration with a functional electron transport chain [Axelsson, 2004].

The activity of LAB in relation to proteolysis and lipolysis is generally weak and so to overcome this situation it is required amino acids, purine and pyrimidine bases and B vitamins [Caplice and Fitzgerald, 1999]. Some genera of LAB also need pantothenic acid and nicotinic acid, and heterofermentative bacteria also need thiamine for their growth [De Vos *et al.*, 2009].

In culture media for growth of LAB are required essential nutrients as fermentable carbohydrates, peptone, and meat and yeast extracts. Furthermore other compounds like manganese salts, acetate and oleic acid esters, among others, are also required since that they are essential or stimulators for most species.

1.8.2. Carbohydrate metabolism

LAB can be divided in two groups, according to dissimilation of glucose and the products formed from the fermentation of sugars [Madigan *et al.*, 2011]. One group, including homofermentative species, produce lactic acid as the sole end product and ferment sugars to pyruvate by the Embden-Meyerhoff-Parnas (EMP) pathway. The other group include heterofermentative species, responsible for the mixed acid metabolism, with formation of lactic acid, CO_2, formate, and ethanol/acetate, by phosphoketolase pathway [König and Fröhlich, 2009].

The selection of each pathway mentioned above is determined at family level and based on this it is possible to classify the bacteria in three types: obligate homofermentative, where the sugars only can be fermented by glycolysis and it comprises the group I lactobacilli; obligate heterofermentative, that only the 6- phosphogluconate pathway is available for sugar fermentation and it includes leuconostocs, group III lactobacilli, oenococci, and weissellas; and facultative heterofermentative LAB, that include group II lactobacilli and most species of enterococci, pediococci, streptococci, tetragenococci and vagococci, and share characteristics of the first two groups [Endo and Dicks, 2014].

Homofermentative. Species responsible for this type of fermentation are called homolactic or homofermenters and include members of various families, as Enterococcaceae, Streptococcaceae and Lactobacillaceae, except for one group in the genus *Lactobacillus* [Endo and Dicks, 2014]. The homofermenters produce about 85% of lactic acid as the sole end product of glucose fermentation [König and Fröhlich, 2009]. The homofermentive species of LAB follows the EMP pathway, with the production of 2 lactates per glucose molecule. In this glycolytic pathway, aldolase splits fructose-1,6-biphosphate into two triose phosphates, being this enzyme the key in this pathway. The triose phosphates are converted to pyruvate and this is reduced to lactic acid by a NAD^+-dependent lactate dehydrogenase, in order to maintain intracellular redox balance [Kandler, 1983; Axelsson, 2004]. During this process two molecules of ATP are formed from one molecule of glucose by substrate-level phosphorylation, subsequently used for biosynthesis (Figure 2A). So, this pathway leads approximately twice as much energy per 1 molecule of glucose as heterofermentative species [Endo and Dicks, 2014].

Heterofermentative. The heterolactic fermentation is conducted by the phosphoketolase pathway, also called 6-phosphogluconate pathway, and it is initiated by the oxidation of glucose-6-phosphate to gluconate-6-phosphate followed by its decarboxylation to yield CO_2, resulting in a pentose-5-phosphate, which is cleaved into glyceraldehyde-3-phosphate and acetyl phosphate. Then glyceraldehyde-3-phosphate is metabolized to lactate (like in homofermentation) and if there aren't additional electron acceptors available the acetyl phosphate is reduced to ethanol via acetyl-CoA and

acetaldehyde [Axelsson, 2004]. In the end, 1 molecule of each lactic acid, CO2, and ethanol are formed from 1 molecule of glucose consumed [Kandler, 1983]. With this type of fermentation one molecule of ATP per molecule of glucose is produced (Figure 2B) [Endo and Dicks, 2014].

An important enzyme in this process is phosphoketolase, which splits the pentose-5-phosphate into glyceraldehyde-3-P and acetyl-phosphate [Axelsson, 2004].

The heterofermenters responsible for this process are leuconostocs, oenococci, weisellas and the group III of lactobacilli and these are more important than the homofermenters, since they produce flavour and aroma components such as acetylaldeyde and diacetyl [Jay, 1992].

This fermentation depends on culture conditions, such as glucose limitation, where the present microorganisms instead of producing only lactic acid, can also produce acetic acid, ethanol and formic acids as end-products. Furthermore the electron acceptors can also change with different conditions [Kandler, 1983]. Thus, different species can use different pathways, depending on conditions and enzymatic capacity [Axelsson, 2004].

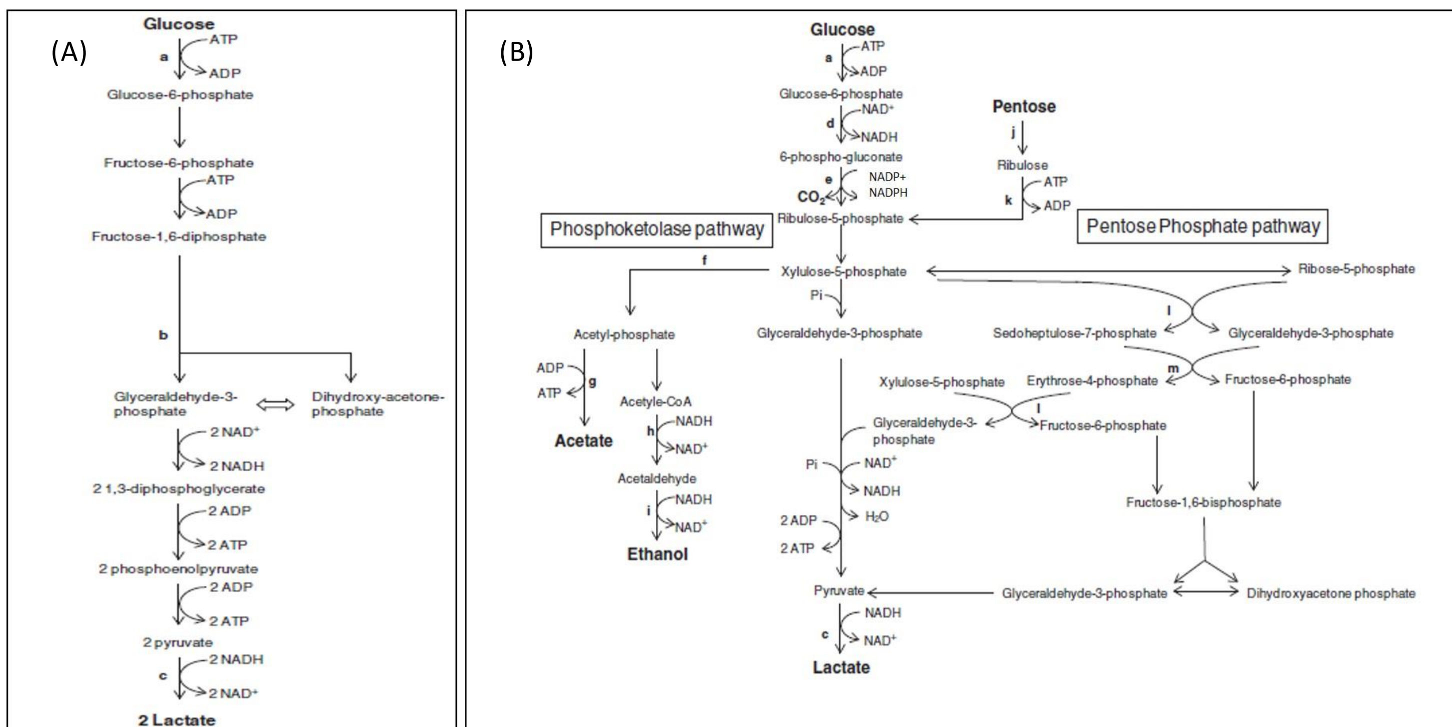

Figure 2. Fermentation pathways of glucose in LAB. (A) homolactic fermentation and (B) heterolactic fermentation. The enzymes used in each pathway are a) glucokinase, b) fructose-1,6-diphosphate aldolase, c) lactate dehydrogenase, d) glucose-6-phosphate dehydrogenase, e) phosphogluconate dehydrogenase, f) phosphoketolase, g) acetatekinase, h) acetaldehyde dehydrogenase, i) alcohol dehydrogenase, j) pentose isomerase ; k) ribulokinase; l) transketolase; m) transaldolase. Taken by Endo and Dicks (2014).

1.9. LAB as probiotic strains

Lactic acid bacteria have been considered beneficial microorganisms and some strains even as health-promoting (probiotic) bacteria [Wright and Axelsson, 2011].

The history of probiotics began with the consumption of fermented foods and, in 1965, Lilley and Stillwell referred for the first time LAB as probiotics in literature. There are many definitions of 'probiotic' term. Etymologically this term is derived from the Greek 'probios' which means "for life" [Florou-Paneri *et al.*, 2013]. The first definition of probiotic as is used today was made by Parker (1974), which defined probiotics as "organisms and substances which contribute to intestinal microbial balance". Since then the definition has been extended to "a live microbial feed supplement beneficial to the host (man or animal) by improving the microbial balance within its body", which was carry out by Fuller (1989) [Savadogo *et al.*, 2006].

According to these definitions, several genus and species are considered probiotic microorganisms. However, only LAB are considered as important in food and nutrition. Microorganisms considered as probiotics are mainly of the *Lactobacillus* genus. The majority of those belong to *Lb. acidophilus*, *Lb. gasseri*, *Lb. helveticus*, *Lb. johnsonii*, *Lb. casei*, *Lb. paracasei*, *Lb. reuteri*, *Lb. plantarum*, *Lb. rhamnosus* and *Lb. fermentum* [Giraffa, 2014].

These microorganisms exert beneficial effects through several mechanisms of action, which have multiple effects on the host. These mechanisms of probiotics rely on their metabolic end products, such as organic acids, which may lower the pH of human gut and consequently inhibit other microbes. Furthermore, the probiotic bacteria compete for sites of pathogenic bacteria, and for available nutrients and produce antimicrobial substances. Thus probiotics (lactobacilli) have potential health effects in several situations preventing diarrheal, colon cancer, lactose intolerance, cholesterol, inflammation, allergic symptoms and improving immune system and having effects on mineral metabolism [Nagpal *et al.*, 2013].

Some strains of LAB can also produce toxins (bacteriocins), which are peptides/proteins with bactericidal activity against some pathogen strains. They improve the shelf-life of foods, promote the inhibition of food spoilage and avoid the addition of chemical preservatives [Florou-Paneri *et al.*, 2013].

A source of probiotics is in fermented food, so the probiotics are used as starters in modern food industry. Thus probiotic foods are those which carry out live cultures of microorganisms, mixed or single, which benefit the host [Montet *et al.*, 2014].

The general safety of lactic acid bacteria is their use without any harmful effects on human health. So these organisms are Generally Recognized As Safe (GRAS) organisms by the Food and Drug Administration [Nagpal *et al.*, 2013].

1.10. LAB in industrial processes

The lactic acid bacteria are a group of microorganisms very important in food and feed industries. They are used in food preservation and they contribute for organoleptic characteristics of foods. Since LAB are able to produce antimicrobials, exopolysaccharides, aromatic compounds, enzymes or nutraceuticals and they have health properties, these bacteria are applied in these industries.

One objective of food industry is to guarantee a long shelf-life of products, so LAB are useful since some strains produce bacteriocins that promote the control of growth of pathogenic microorganisms. As a consequence, the LAB with these characteristics emerge as protective cultures in fermented meats, fermented vegetables and in dairy products, conserving the food. Other characteristic of LAB useful in food industry is the capacity of produce exopolysaccharides, which are known to increase the viscosity and firmness, contributing to the texture that promote the sensation of low fat products. Furthermore, amylase is also produced by some bacteria and can be used in the improvement of texture of food, which is beneficial in bakery industry [Giraffa, 2014].

Additionally, lactic acid bacteria have been useful for modification of the organoleptic characteristics of foods, such as the flavour, by producing aromatic compounds (in sourdough with lactate/acetate, in kefir with ethanol, in butter with diacetyl and in yogurt with acetaldehyde), and texture, with their proteolytic and pepetydolitic activities, contributing for example, to ripening of cheeses [Das and Goyal, 2012].

These bacteria are also used as bio-preservative antimicrobial agents, since they produce lactic acid used as acidulant, flavourings and as inhibitors of pathogenic microbiota. Thus, there is a greater interest in these bio-preservatives with safe association, since they are more preferred than chemical additives currently used [Das and Goyal, 2012].

Furthermore, lactic acid bacteria have also an important role in pharmaceutical industry for synthesis of chemicals. Metabolic engineering can be used in these strains to improve biotechnological characteristics in production of nutraceuticals. Strategies of metabolic engineering can contribute to alteration of end product in lactic acid fermentation, through other compounds (aromatics, or sugars) with preferential characteristics [Giraffa, 2014].

1.11. Objectives of this study

This study emerged from a partnership project (BioPepperTec) between Mendes Gonçalves Company and Lab Bugworkers|M&B-BioISI from FCUL, which aims to explore the biological fermentative processes of peppers producing new distinct products at organoleptic and nutritional levels. By using a full exploitation of peppers, fermented pepper paste and pepper whey were co-produced from lactic acid fermentation, with subsequent production of pepper whey wine and pepper whey vinegar, by an alcoholic and acetic two-step fermentation. From spontaneous fermentation of pepper pastes, it was required the isolation and characterization of lactic acid bacteria, to be used in biotechnological processes, controlled from a microbiological point of view. Furthermore, they bacteria were also intended to create an optimized starter's bank to use in subsequent controlled fermentations in order to

obtain pepper pastes with better organoleptic characteristics, ensuring the repeatability and reproducibility required. For this goal a tool-kit of new microbiological and molecular methodologies should be implemented for integrated monitoring and control of industrial fermentations.

2. MATERIAL AND METHODS

2.1. Raw material

The red and green habaneros and hot peppers were obtained from different local markets in Lisbon. Bell peppers and chillies were directly achieved from producers in Golegã by Mendes Gonçalves Company. All peppers were carefully selected, without apparent damages and were stored at 4ºC until processed to produce pepper paste.

2.2. Culture media

The growth medium for lactic acid bacteria was Mann Rogosa and Sharp broth (MRS) prepared by dissolving 55.3 g of dehydrated medium in 1 L of distilled water and sterilized in an autoclave at 110ºC for 45 minutes.

This medium is constituted (g/L) by polypeptone (10), meat extract (10), yeast extract (5), glucose (20), tween 80 (1.08), dipotassium phosphate (2), sodium acetate (5), ammonium citrate (2), magnesium sulphate (0.2) and manganese sulphate (0.05) at pH 6.5.

During the fermentation process was added 0.1 mg of cycloheximide (CYC; Sigma) per 1 mL of medium, to prevent growth of yeasts or fungi. The MRS solid medium was prepared similarly to MRS broth but with the addition of 1.5% (w/v) of bacteriological agar.

For the isolation of yeasts and fungi the YEPGA medium was used. This is constituted (g/L) by yeast extract (10), peptone (peptic digest of meat) (20) and glucose (20).

All compounds and media were from Biokar diagnostics, except otherwise stated.

2.3. Preparation of pepper paste for the spontaneous fermentation

Two techniques were applied in preparation of pepper paste. In both, seeds and stems of peppers were removed and peppers were cut in little pieces and washed with tap and autoclaved water to be minced.

To the first method of preparation of pepper paste, the peppers were minced with mortar and pestle and after this, it was made a resuspension in 20 – 40 mL of MRS with 0.01% of cycloheximide, to prevent the growth of fungi and yeasts, and 0% or 3% of NaCl in an Erlenmeyer.

Using this method were performed five different batches with different raw material (one batch with a mix of red and green habaneros peppers much mature; other with hot pepper much mature; a batch just with green habaneros peppers; and other with just red habaneros peppers; and the last with hot peppers).

The other method used to obtain the pepper paste was achieved using a blender to homogenise the little pieces of peppers and then it was directly added with 5%, 10%, 12% or 15% of NaCl in a beaker to initiate the spontaneous fermentation. With this method to prepare the pepper paste were used chillies, bell peppers and hot peppers.

The surplus was frozen in portions of 100 g at -20ºC.

2.4. Isolation of bacterial strains from spontaneous fermentation of peppers to create a culture collection

The resuspensions in MRS medium in spontaneous fermentation were inoculated by spreading on plates of MRS + CYC 0.01% each 24 h, 48 h, and 72 h after performing serially dilutions (10^0 , 10^{-1}, 10^{-2} , 10^{-3}, 10^{-4}, 10^{-5} e 10^{-6}) and incubated aerobically at 28ºC for 15 days.

The rest of batch of fermentations from spontaneous fermentations (without media on batch) were incubated also at 28ºC and inoculated on plates of MRS with 0.01% of CYC, but the platting occurred only after three days of the beginning of spontaneous fermentation. On batch of fermentation with 5% of NaCl, the serial dilutions 10^0, 10^{-3}, 10^{-5} and 10^{-6} were made and plated on MRS agar. On batch with 10%, 12% and 15% of NaCl the dilutions 10^0, 10^{-2} and 10^{-4} were performed before plating on MRS. All dilutions were performed in saline solution (NaCl 0.8%).

For isolating presumptive mesophilic lactic acid bacteria, colonies with different morphology from MRS plates of various dilutions were isolated, and this process was repeated until a pure culture was obtained by streaking and subculturing on fresh MRS without antibiotic, being then subjected to a preliminary identification.

Isolates were preserved in MRS broth using 20% (v/v) glycerol at -80ºC.

2.5. Phenotypic characterization of lactic acid bacteria

The phenotypic characterization of pure isolates was done according to their physiological and biochemical characteristics. So, after pure cultures, the colonies were subjected to phenotypic tests to confirm the characteristics of lactic acid bacteria like coccus or rods, negative catalase, oxidase and KOH negative and positive gram staining. Some strains were also grown in MRS with 6.5% of salt and 2.2% of agar to select and exclude *Enterococcus*, since they are distinguished from the other lactic acid bacteria because they grow with this percentage of NaCl.

2.6. Molecular typing and identification of lactic acid bacteria

2.6.1. DNA extraction

Genomic DNA from each strain of presumptive lactic acid bacteria was extracted by guanidine thiocyanate method adapted from Pitcher *et al.* (1989). The cultures were grown in MRS agar aerobically at 28ºC overnight and they were ressuspended on eppendorf of 2 mL with 250 µL of lysis buffer (50 mM Tris; 250 mM NaCl; 50 mM EDTA; 0.3% SDS; pH 8.0). It was added 100 µL of microspheres, following by agitation in vortex 2 min. Tubes were incubated at 65ºC for 30 min. Cells were lysed with 250 µL of GES solution, prepared as described in Pitcher *et al.*, after other 2 min of agitation in vortex. After extraction with equal volume of chloroform : isoamylic acid (24:1), the supernatant was collected after centrifugation and isopropanol was added in an equal volume. The tubes were mixed by inversion and the DNA was centrifuged at 18 000 g for 10 min. Pellets of DNA were washed in 1 mL of cold ethanol 70%, dried and solubilized in 50 µL of TE.

2.6.2. M13 and $(GTG)_5$ PCR fingerprinting

Genomic DNA extracted from strains was used as a template for PCR fingerprinting. Since some strains are not distinguishable with one type of PCR fingerprinting, were used two methods of PCR fingerprinting, using M13 (5' GAG GGT GGC GGT TCT 3') and $(GTG)_5$ (5' GTG GTG GTG GTG GTG 3') as sole primers in separate amplifications [Gevers *et al.*, 2001; Rossetti and Giraffa, 2005]. PCR reactions were carried out in 25 µL amplification mixtures in both T1 and T gradient Termocyclers (Biometra) with 1x of PCR buffer, 3 mM of $MgCl_2$, 1 pmol μL^{-1} of primer, 0.2 mM of dNTPs, 1 U of Taq Polymerase, 1 µL of total DNA and water. The negative control used did not contain DNA. One cycle of 95ºC for 5 min was followed by 40 cycles of 95ºC for 1 min (denaturing), 50ºC for 2 min (annealing) and 72ºC for 2 min (extension). Final extension occurred at 72ºC for 5 min. The samples was kept at 4ºC until the handling.

2.6.3. Gel electrophoresis and DNA visualization

Amplification products from fingerprinting PCR (M13 and GTG_5) were subjected to electrophoresis performed in 1.2% (w/v) of agarose gels in 0.5 x TBE buffer. Gels were prepared by suspending agarose in TBE buffer, and after this heating in a microwave to dissolve completely. Subsequently, dissolved agarose was kept at 55ºC to stabilize before using. The products of PCR were mixed with PCR loading buffer, and after the gel polymerized, loaded into wells in the gel and run at 90 V for 3 h. A DNA molecular mass marker (1 kb plus DNA ladder) from Invitrogen was used as a standard. After run, the gels was stained with a solution of ethidium bromide for 10 minutes, visualized under UV light and photographed using the Alliance 4.7 UV transiluminator (UVITEC, Cambridge) using the Alliance software (version 15.15).

2.6.4. Analysis of fingerprinting profiles

The digitised images were analysed and processed using the Bionumerics software. To group the fingerprints was used the Pearson correlation coefficient as similarity measure and the unweighted pair-group method using arithmetic averages (UPGMA) as clustering algorithm. The profiles M13 and $(GTG)_5$ PCR fingerprinting of bacterial isolates were evaluated and combined to obtain a unique dendrogram. For each primer, 10% of duplicates were performed to assess the reproducibility.

2.6.5. Molecular identification by 16S rRNA gene sequencing

For identification of lactic acid bacteria by sequencing was applied a specific PCR where the target is 16S rRNA gene. PA forward primer (5' AGAGTTTGATCCTGGCTCAG 3') and 907R reverse primer (5' CCGTCAATTCMTTTRAGTTT 3'), were used amplifying a 900-bp DNA fragment. The final volume of reaction was 50 µL, consisting of 1x of PCR buffer, 2 mM of $MgCl_2$, 1 pmol of each primer, 0.2 mM of

dNTPs, 1 U of Taq Polymerase, 1 µL of total DNA and 38.8 µL of water. The amplification program is composed by one cycle of 94ºC for 5 min, followed by 35 cycles of 94ºC for 1 min (denaturing), 55ºC for 1 min (annealing) and 72ºC for 2 min (extension). Final extension occurred 5 min at 72ºC. The negative control used did not contain DNA. The reagents used in PCR reaction were obtained from Invitrogen. Visualization of amplicons was performed as described in 2.6.3. but using 0.8% (w/v) agarose gel and 90 V for 1h.

The purification of amplified DNA (50 µL) was conducted using JETquick PCR product purification spin kit (Genomed). All the solutions and columns of silica required for this process were supplied in the kit. The procedure followed the indications of kit brochure. Sequencing was performed by service acquisition at Biopremier.

The delivered sequence data in FASTA format was analysed and compared with NCBI database using BLAST algorithm.

2.7. Physiological characterization of culture collection

For each assay, the selected isolates of lactic acid bacteria were cultivated in approximately 5 mL of MRS broth in tubes of 15 mL (falcon-like tubes) at 28ºC overnight in aerobic conditions. To determine which strains have the best characteristics, several experiments were performed using these pre-cultures.

2.7.1. Growth curves

To analyse the growth of previously purified isolated, growth curves were performed. So, bacterial growth was determined spectrophotometrically by measurement of optical density at 600 nm by UV 1101 Biotech Spectrophotometer (WPA); for all isolates growth began with the same concentration. The spectrophotometer was calibrated against sterile MRS medium of the same composition as was used for bacterial incubations. With high culture densities, samples were diluted on media to give OD_{600} readings in range of 0.005-0.5.

After overnight incubation pre-cultures, the absorbance was measured to determine the inoculum volume needed to obtain an initial OD_{600} of 0.005 in the culture.

For these experiments were used triplicate cultures in 250 mL Erlenmeyer flasks with a medium volume of 100 mL.

During the assay, samples were collected each hour and readings of OD_{600} were made, as well as the measurement of pH. Before measurement of pH the Eppendorf tubes containing 1 mL samples were centrifuged by 10 minutes at 18 000 g and the supernatant kept for reading of pH. The pH was determined by a pH-meter equipped with a glass microelectrode (Denver Instrument model 15) which was calibrated against standard buffer solutions at pH 4.0 and 7.0.

2.7.2. Tolerance to pH, NaCl and temperature

Conditions to determine growth parameters were performed in 100-well microplates on Bioscreen apparatus (Thermo Scientific). Several culture media were used to determine the tolerance and ability of LAB to grow in different concentrations of salt, pH and temperature. For that, 15 MRS broth media were used combining different pH (4.5, 5.5 and 6.5) and NaCl concentrations (0, 3, 6, 12 and 15%), and different temperatures (25, 28 and 30ºC), where set up in Bioscreen for each test. pH of each media was adjusted with a hydrochloric acid solution.

Pre-cultures for microplates assays were prepared as described above. The volume of the pre-culture used for the inoculum was calculated so that the inoculum preparation had an initial OD_{600} of 0.3. The volume of inoculum was centrifuged at 18 000 g during 10 minutes and ressuspended in 1 mL of appropriate MRS medium in tubes. Then 10 µL of inoculum was transferred to wells, which were filled with 290 µL of each experimental medium. In this assay duplicates were realized. The OD_{600} nm was measured every 15 minutes for 12 h. The temperature was also adjusted to the desired temperature for testing.

The positive control used was MRS medium pH 6.5 without NaCl and the negative control for each condition was the respective medium without inoculum. Data from microplate experiments were processed using Microsoft Office Excel 2013.

2.7.3. CO_2 production from glucose

Gas (CO_2) production from glucose was determined in assay tubes with 7 mL of MRS broth, containing inverted Durham tubes, which had been autoclaved for 45 minutes at 110ºC. After growth, the isolates were inoculated with a calibrated loop (10 µL) of cells and incubated for 48 h at 28ºC. It is considered a positive result if the Durham tube contains gas inside and negative result if not.

2.7.4. Enzymatic assays for determination of sugars and metabolites

The configuration and concentration of each of the isomers of lactic acid produced from glucose and the consumption of glucose and fructose were determined enzymatically in the cell-free supernatant of cultures collected to characterize the metabolism of strains.

The cultures were grown in MRS broth for 12 h and samples were collected every hour. The times for analysis were 2 h, 4 h, 6 h, 8 h and the final point. To the samples obtained from different pepper fermentations the samples were collected at 0 h, 5 h and 12 h. Triplicates were performed for each one. After this, the samples of both sources were centrifuged 10 minutes at 18 000 g to collect the supernatant.

These tests were performed using several enzymes that favours the reaction to produce NADH or NADPH, which is measured by the increase in absorbance at 340 nm, in microplate reader (Anthos Zenyth 3100). The analysis were conducted using Megazyme Kits in 96-well microplate.

2.7.4.1. Calibration curves

For each kit a calibration curve was required to convert the value of absorbance into a concentration. The calibration curve was constructed with a standard solution of the kit that allowed, through various dilutions with sterile water, the obtention of a set of various concentrations within the linearity of each kit.
To each curve was determined an equation to express the concentration of products and each curve had a coefficient of determination (R^2) value in a range of 0.95-0.99.

2.7.4.2. Determination of D- and L- lactic acid

For the quantification of D- or L- lactic acid two enzyme reactions were required, carried out by D- or L-lactate dehydrogenase (D-LDH or L-LDH) and D-glutamate-pyruvate transaminase (D-GPT).
The amount of NADH formed in this reaction is stoichiometric with the amount of D- or L- acid lactic. So, it is the NADH which is measured by the increase in absorbance at 340 nm.
The procedure was followed according to kit descriptions and incubation times were performed at 25ºC. In some cases were required dilutions performed with sterile water, since linearity of the reactions is between 0.5 – 3 µg of D-lactic acid per well in 10 µl – 150 µl sample volume and 0.1 – 3 µg of L-lactic acid per well in 10 µl – 200 µl sample volume. The solutions and enzymes required were supplied by the kit.

2.7.4.3. Determination of glucose and fructose

Consumed glucose was also dosed to understand the metabolism of lactic acid bacteria and their yield. The principle of dosage is also driven by action of two enzymes, hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6P-DH). The NADPH formed from this reactions, was measured at 340 nm since it is stoichiometric with the amount of D-glucose.
In some samples collected was necessary to make a dilution, because the limit of detection of the kit is between 0.1 – 8 µg of glucose per well in 10 µL – 200 µL sample volume. ´
While in supernatant obtained from growth curves only the glucose was analysed, in samples from fermentation both sugars, glucose and fructose, were measured. To detect the fructose another enzyme supplied by the kit was required (phosphoglucose isomerase - PGI). One more time the NADPH was measured. The procedure was followed as indicated by brochure kit.

2.7.4.4. Data analysis

After getting the data from microplate reader (Anthos Zenyth) these were analysed in Microsoft Office Excel 2013. Concentration of products were determined by using the calibration curve equations and the dilution factor, if applicable.

2.8. Pepper fermentation assays at lab-scale

2.8.1. Preparation of pepper paste for the controlled fermentations

The main procedure for production of this pepper paste was the same that in pepper paste of spontaneous fermentation, i.e. also the seeds and stems of peppers were removed and peppers were cut. Before this, the pieces of pepper were washed with tap water and every type of pepper (bell pepper and hot pepper) was dipped in sterile water with bleaching (10 drops per litre) during 30 min. Then the peppers were washed with autoclaved water and strained in horizontal laminar flow chamber. The peppers were minced in blade mixer and weighted in portions of 100 g and they were placed in storage jars previously sterilized. Some pastes were added with 5% NaCl. The remnant was frozen in portions at -20ºC.

2.8.2. Inoculation of Starter cultures

For the preparation of starter cultures, strains were grown in MRS broth like described in section 2.7 during 24 h, and after this the OD_{600} was measured to determine the inoculum volume to obtain ca. 9.0 log CFU ml^{-1} in the final volume of pepper paste. The inoculum was centrifuged during 10 min at 18 000 g and washed in NaCl 0.8%.
After inoculation, the pepper paste was incubated at 28ºC during 12 days and the kinetics of growth and acidification were determined. Growth was monitored by platting on MRS agar incubated aerobically at 28ºC for approximately 3 days and a daily sample of 2 mL was taken and stored at -20ºC for subsequent analysis.

2.8.3. Microbiological analysis

Colonies from fermentation isolated in MRS agar and incubated at 28ºC were characterized in relation to the form and Gram staining. Its DNA was extracted by the method of Pitcher *et al.* (See section 2.6.1) to subsequent PCR fingerprinting to confirm if starter culture remains along the fermentation.
Some fungi were also isolated in YEPG agar and microscope slides were prepared with cotton blue staining to characterize and identify, through dichotomous keys.

2.9. Sensory analysis

Organoleptic analysis of fermented bell and hot peppers was performed by a panel of tasters (including trained and untrained elements) using a blind test. The samples were identified by a code and they followed a certain order. First the bell peppers without NaCl, second the bell peppers with 5% of NaCl, third the hot peppers without salt and at last the hot peppers with 5% NaCl. Between each paste the panel of tasters used grapes and yoghurt sauce to cut the flavour of each one. The group consisted in 10 people of both sexes and different ages (27 – 40) and they tasted the peppers in groups of two or three. Visual aspect, flavour and aroma of fermented peppers were the attributes evaluated by tasters.

Each parameter was evaluated using a 9-point hedonic scale, where 1 means dislike extremely and 9 means like extremely. These parameters were selected as described by Seseña *et al*. (2001).

2.10. Statistical analysis

To compare and group isolated strains from different spontaneous fermentations, a Principal Component Analysis (PCA) of the NAUCs (Net Area Under Curves) observed in different conditions was performed. The PCA consists in a transformation of data into variables, designed by Principal Components (PC), that allows to reduce the complexity of the original dataset and to group strains based on their similarity.

PCA was made in NTSYS software (2.21 version) from a matrix of variables (NAUC in different conditions) vs strains obtained in Microsoft Excel 2013. With NTSYS software, 2D projections with the distribution of strains in different PCs (PC1, PC2 and PC3) were obtained.

3. RESULTS AND DISCUSSION

3.1. Total viable cells population on spontaneous fermentation

Different samples of peppers (chillies, green or red habaneros peppers and hot peppers) were analysed for microbial mesophilic counts. The results of the counts of microorganisms present on pepper paste with or without salt, during fermentations were similar in every pepper pastes. In all pepper pastes, the population were in the range of 10 to 10^9 cfu g^{-1}.

3.2. Identification of lactic acid bacteria

From different batches of spontaneous fermentations, a total of 125 strains were isolated in the MRS plates from different pepper pastes.
In the first batch of fermentation with a mix of red and green habaneros peppers (in an advanced state of maturation) it was possible to obtain 10 isolates in absence of salt and 8 with 3% of salt, while in mature hot peppers were isolated 9 (0% salt) and 8 strains (3% of NaCl).

In other pepper paste from hot peppers were isolated 4 strains without NaCl and 13 in presence of 3% NaCl. From red habaneros without NaCl it was possible to isolate 14 strains and 10 with 3% NaCl. Relatively to green habaneros 12 and 15 isolates were obtained, without and with salt, respectively. In the last batches, which performed with 5% of salt, were isolated 6 from a variety of hot peppers, 8 from chillies and 1 from bell peppers and in batches with 10% of NaCl, 3 strains were isolated, 1 from chillies and 2 from hot peppers. Finally it was also isolated one strain from hot pepper paste with 12% of salt.

Amongst the isolated strains, 80 had characteristics of LAB, being Gram-positive and negative for catalase, oxidase and KOH. The shape of cells was rod or coccus and some were arranged in pairs, chains, isolated or grouped. From results of test of production of gas from glucose, the strains were divided in hetero- and homo- fermentors, being the mostly homofermentative strains, with only two heterofermenters. Seventy three strains were subjected to a test (growth with 6.5% NaCl) to exclude *Enterococcus* spp., since they would not be safe to use in pepper pastes, where 46 presented growth (potential *Enterococcus* spp.) and 10 presented only a little growth in 6.5% of NaCl. From 46 that obtained a positive test to *Enterococcus* spp., from 10 that presented a little growth in 6.5% of NaCl and from 17 that did not grow were selected 10, 2 and 6, respectively.

Other 10 hypothetic LAB isolated by Mendes Gonçalves Company were also characterized to confirm the characteristics of lactic acid bacteria. These strains were obtained from natural microbiota of different types of peppers, not subjected to a fermentation. Only one strain did not show these characteristics, since it was positive for catalase. The remaining were rod or coccus, arranged in pairs, chains, or isolated.

After being characterized phenotypically, the strains with similar characteristics, obtained from fermentations, were grouped and after this were subjected to genotypic tests.

Total DNA from 25 LAB strains isolated from different phases of the fermentation of different pepper pastes and 9 LAB from microbiota of different peppers was used to generate M13 and $(GTG)_5$ fingerprintings with different patterns (bands ranging from 200 bp to 5000 bp), which were used for sub-grouping of strains. By software Bionumerics, the profiles obtained with primers M13 and $(GTG)_5$ were included in a dendrogram (obtained by the average of two similarity matrices) shown in Figure 3, being the level of minimum reproducibility of both methods about 95%.

From the different clusters obtained were selected strain(s) of some clusters to be identified by partial sequencing of the 16S rRNA gene. The identified species were *Enterococcus faecallis* (1 isolate), *Lactococcus lactis* (3), *Enterococcus gilvus* (1), *Enterococcus gallinarum* (1), *Enterococcus casseliflavus* (3), *Enterococcus mundtii* (1), *Leuconostoc citreum* (2), *Lactobacillus brevis* (1), *Lactobacillus hilgardii* (1), *Lactobacillus plantarum* (2) and *Weisella cibaria* (1).

The strains LAB 009, LAB 005 and LAB 006 isolated by Mendes Gonçalves Company are grouped with the strains *Leuconostoc citreum* (LAB 004 and LAB 011) at a similarity level of 70%. Three strains obtained in spontaneously fermented peppers are included on cluster of *Enterococcus faecalis* (LAB 023) above 90% of similarity level. The cluster of *Lactobacillus plantarum* (LAB 012 and LAB 013) with approximately 80% of similarity include three strains isolated from spontaneous fermentations of chillies with 5% of NaCl. The *Weissela cibaria* (strain LAB 014) is found in a cluster with another strain with 100% of similarity. The strains of *Enterococcus casseliflavus* (LAB 063 and LAB 076) are found in the same cluster of strain LAB 075. However it is possible to find other *E. casseliflavus* strains in a distant cluster, which can be explained by huge differences in gel staining intensity. Three *Lactococcus lactis* strains obtained from microbiota original of peppers (LAB 002 and LAB 008) and from hot peppers (LAB 047) are in the same cluster with 60% of similarity. Observing the dendrogram it is also noticeable that there is a big cluster that includes a strain of *E. mundtii* with aproximatelly 80% of similarity.

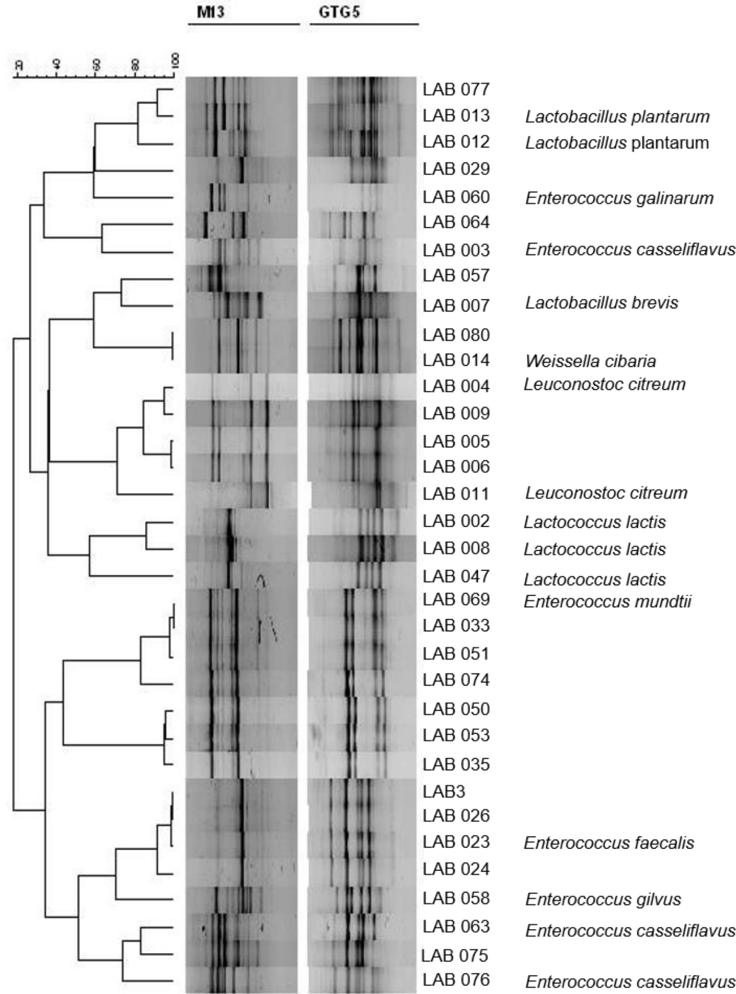

Figure 3. Dendrogram obtained by combined M13 and (GTG)$_5$ fingerprintings for the isolates obtained from habaneros peppers, hot peppers and bell peppers, with 0, 3 or 5% of NaCl. Identification of strains by partial sequencing of 16S rRNA gene. Cluster analysis was based on the Pearson coefficient and unweighted pair group with arithmetic average.

3.3. Evaluation of strain growth in MRS medium

In order to allow the verification of ability to grow in MRS medium were followed growth curves of 8 different strains, including two strains of each species (*Enterococcus* not included), with exception of *Lactobacillus brevis* and *Lactobacillus hilgardii* with only one strain. During 12 h of incubation in MRS broth at 28ºC, the latency time (λ h), the biomass production (OD_{max}) and the growth rate (μ) were evaluated to select the best strains to be used in controlled fermentation (Table 1). Beyond these characteristics also the final pH was measured, since acidification is an essential characteristic of LAB starter cultures, since lower values of pH lead to a better ability to prevent spoilage [Hitendra *et al.*, 2016].

Table 1. Characteristics of the growth curves of the selected strains in MRS medium. The values correspond to growth rate (μ h^{-1}), latency time (λ h^{-1}), maximum optical density (OD_{max}) and final pH. Growth was performed at 28ºC without shaking, using an initial OD of 0.005.

Strains	μ (h^{-1})	λ (h^{-1})	OD_{max}	pH
Lactococcus lactis **LAB002**	0.394	3	2.68	4.55
Lactococcus lactis **LAB008**	0.287	2	1.96	4.66
Leuconostoc citreum **LAB004**	0.344	1	2.33	4.55
Leuconostoc citreum **LAB 011**	0.311	3	2.48	4.64
Lactobacillus brevis **LAB007**	0.211	2.67	2.19	4.99
Lactobacillus hilgardii **LAB010**	0.127	3.33	0.39	6.04
Lactobacillus plantarum **LAB 012**	0.306	4	4.69	4.39
Lactobacillus plantarum **LAB013**	0.313	4	4.72	4.37

From these results, *Lc. lactis* LAB 002 was the strain with the highest growth rate, but on the other hand was not the strain with higher OD_{max}, being this characteristic found in two strains of *Lactobacillus plantarum*. Although *Lb. plantarum* strains had a longer time of latency, these strains are able to adapt to the environment, reaching high biomass. Furthermore these latter strains also presented the best capability in acidification of MRS medium, reaching values of 4.35±0.4. The *Lb. hilgardii* was the strain with worst capacity to growth and to acidify the medium.

The values of growth rate between strains of the same species are proximal, pointing to a correlation between strains, except for *Lactococcus lactis*, since strain LAB 008 does not have the maximum growth rate such as LAB 002.

3.4. Growth of LAB strains at different pH, temperature and NaCl concentrations

The eight strains mentioned above were used for the evaluation of the ability to grow in different conditions of pH, concentrations of NaCl and temperature, once these factors affect the growth of LAB. During 12 h the OD was measured to evaluate the growth behaviour of each strain and the NAUC (Net Area Under Curve) was calculated (Figures 4 and 5).

The majority of strains grew better at pH near of neutral (6.5), in absence or with low concentration of NaCl (3%) and at higher temperatures (28ºC or 30ºC). Concentrations of salt above 3%, in most of cases, inhibit the growth of LAB such as temperatures lower than 28ºC do not favour their growth.

The strain that presented the maximum growth was *Leuconostoc citreum* LAB 004 at 30ºC, 0% NaCl and pH 6.5 and the strain *Lactobacillus hilgardii* did not grow in any condition.

Both strains of *Lactobacillus plantarum* grow better with higher temperatures (30ºC) comparing with other temperatures in study, while in other strains there are not a big difference between 28ºC and 30ºC in their growth (Figure 5). At 25ºC there was high variability among replicates and no clear pattern of differentiation could be identified.

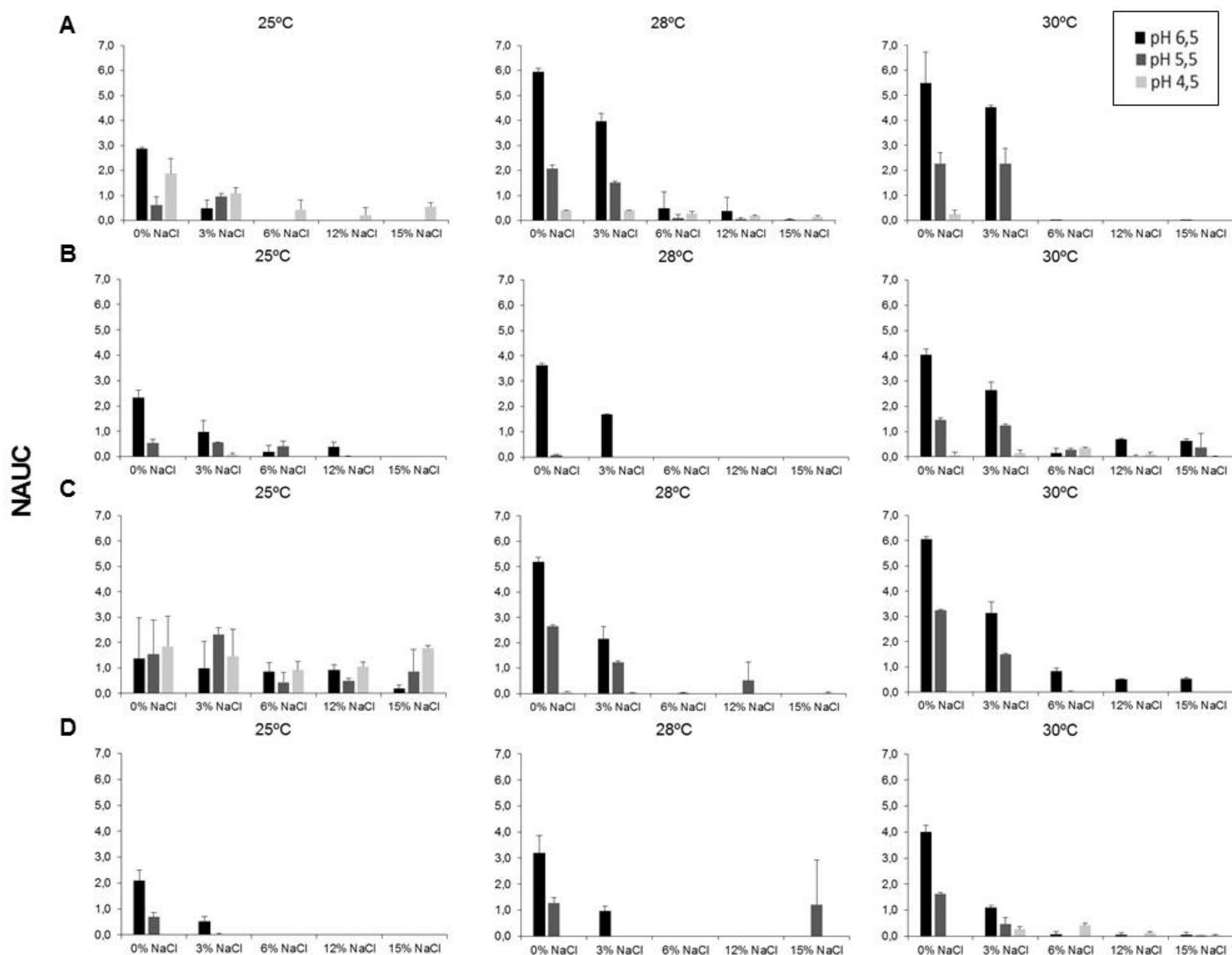

Figure 4. Net area under curve (NAUC) of growth of selected strains. (A) *Lactococcus lactis* LAB 002, (B) *Lactococcus lactis* LAB 008, (C) *Leuconostoc citreum* LAB 004, and (D) *Leuconostoc citreum* LAB 011 in MRS without agitation at different temperatures (25, 28 and 30ºC), pHs (4.5, 5.5 and 6.5) and different concentrations of NaCl (0, 3, 6, 12 and 15%). Growth was evaluated by OD at 600 nm during 12 h. The control situation corresponded to the first bar in black in each graphic. Results are means of two independent trials, each carried out in duplicate. Error bars represent the standard deviation.

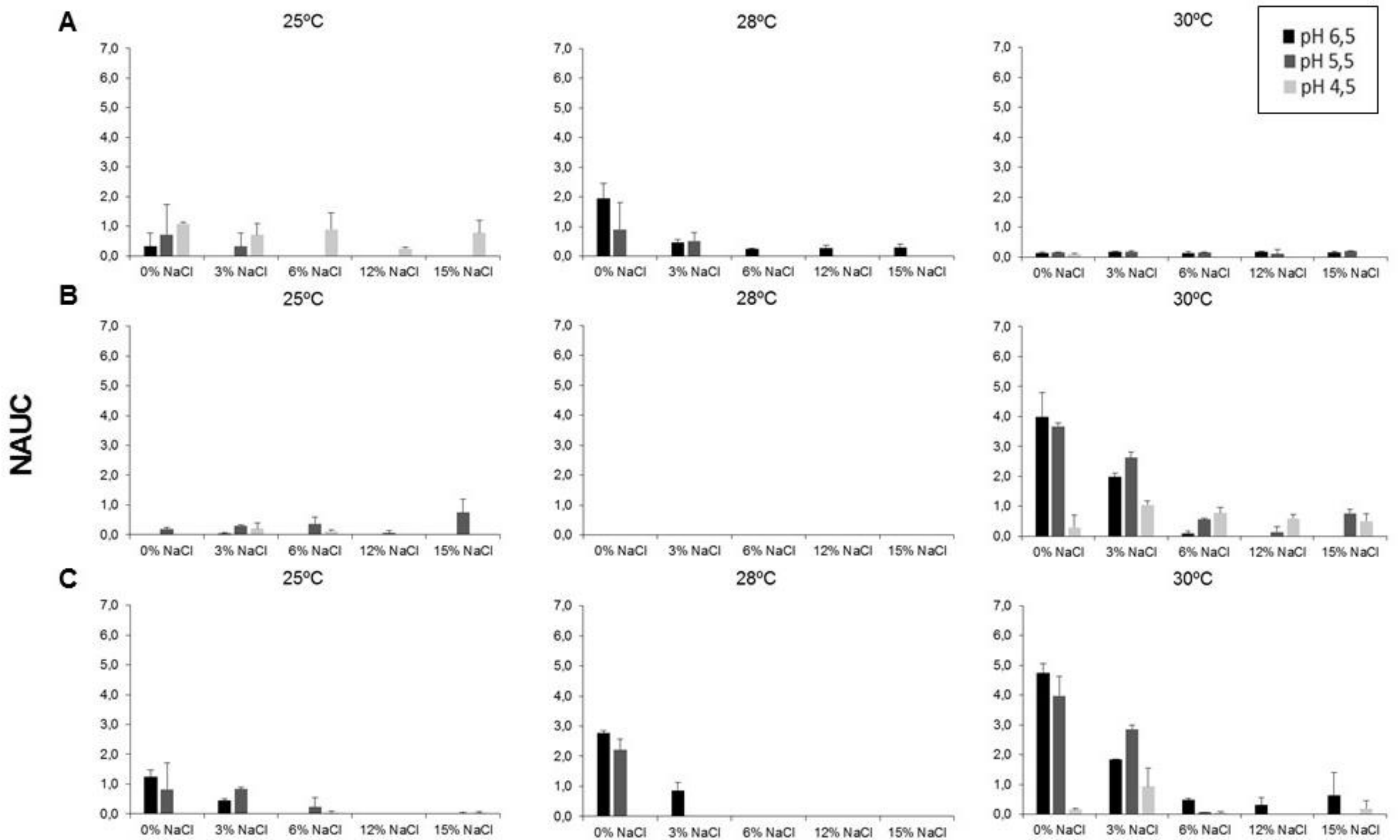

Figure 5. Net area under curve (NAUC) of growth of selected strains. (A) *Lactobacillus brevis* LAB 007, (B) *Lactobacillus plantarum* LAB 012, (C) *Lactobacillus plantarum* LAB 013 in MRS without agitation at different temperatures (25, 28 and 30ºC), pHs (4.5, 5.5 and 6.5) and different concentrations of NaCl (0, 3 ,6 ,12 and 15%). Growth was evaluated by OD at 600nm during 12 h. The control situation corresponded to the first bar in black in each graphic. Results are means of two independent trials, each carried out in duplicate. Error bars represent the standard deviation.

Similar studies revealed that *Lb. brevis* have a higher survival in lower pH (pH 3) comparing with *Lb. plantarum*, such as in this study with temperatures of 25ºC [Garcia *et al.*, 2016]. However with higher temperatures *Lb. plantarum* showed a better growth. Furthermore, both strains of *Lactobacillus* studied by Garcia *et al*. (2016) had higher viable counts with pH near neutral (5 and 7.2). Nevertheless, the present work also revealed a better growth with higher pH.

So, from these results there is an evident effect on growth with the different values of each parameter used, where is observable that when pH decrease and NaCl concentrations increase, the growth is lower. In relation to temperatures this evidence is weak, since the strains grow at both 28ºC and 30ºC, with exception of *Lactobacillus plantarum* strains that grows better at 30ºC.

For a better understanding of growth of microorganisms and of how they group together, a Principal Component Analysis (PCA) was also made. The groups formed are distributed in 2D graphs, representing each one a projection on a plane defined by the principal components (PC). The principal component analysis with all strains revealed five groups, as depicted in Figure 6 (PC1 x PC2 graph) and Figure 7 (PC1 x PC3 graph). Since the cumulative percentage of variation retained by the set of 3 PCs was 76.6%, these projections represent a good snapshot of the global variability.

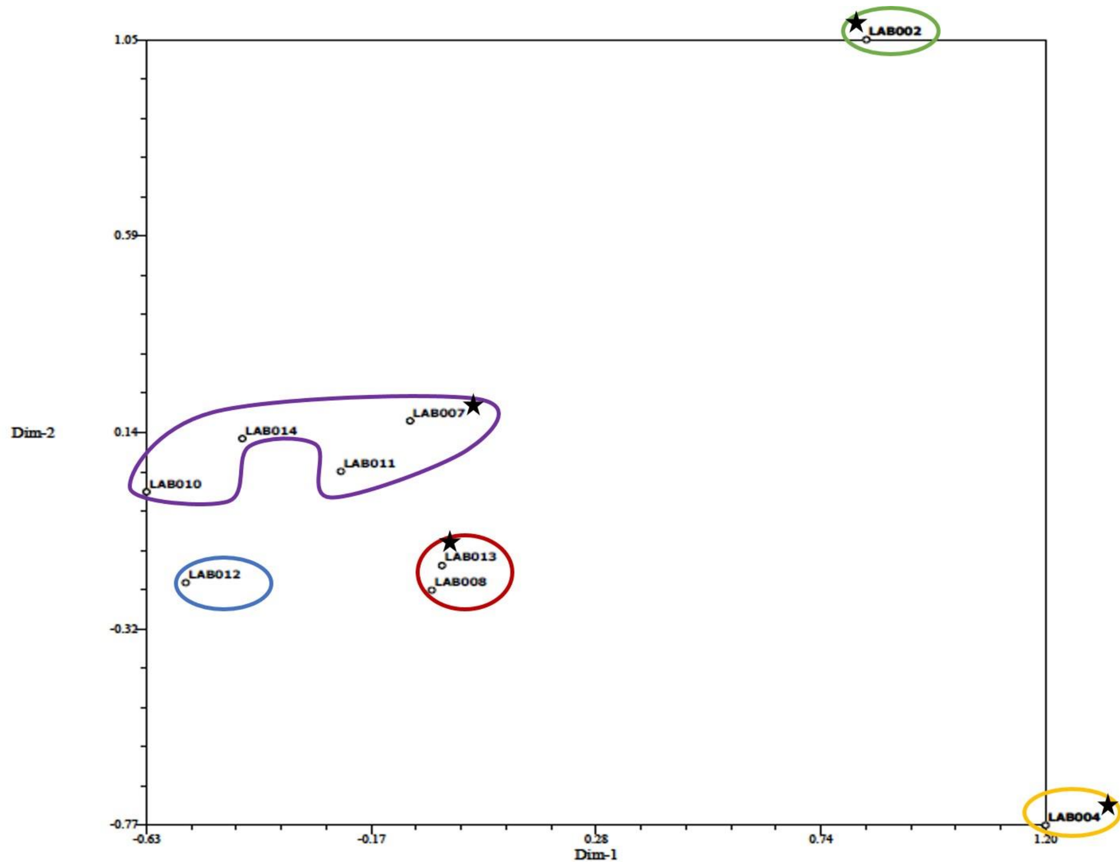

Figure 6. PC1 x PC2 projection of PCA of strains isolated from spontaneous fermentation. Distribution on space of strains defined by PC1 (Dim-1) and PC2 (Dim-2). The matrix used in PCA was 45 by 9 (45 conditions by 9 strains), being the variation associated to PC1 of 38.96% and to PC2 of 32.37%. Each circle correspond to a defined group of strains. Stars represent the strains selected for controlled fermentations.

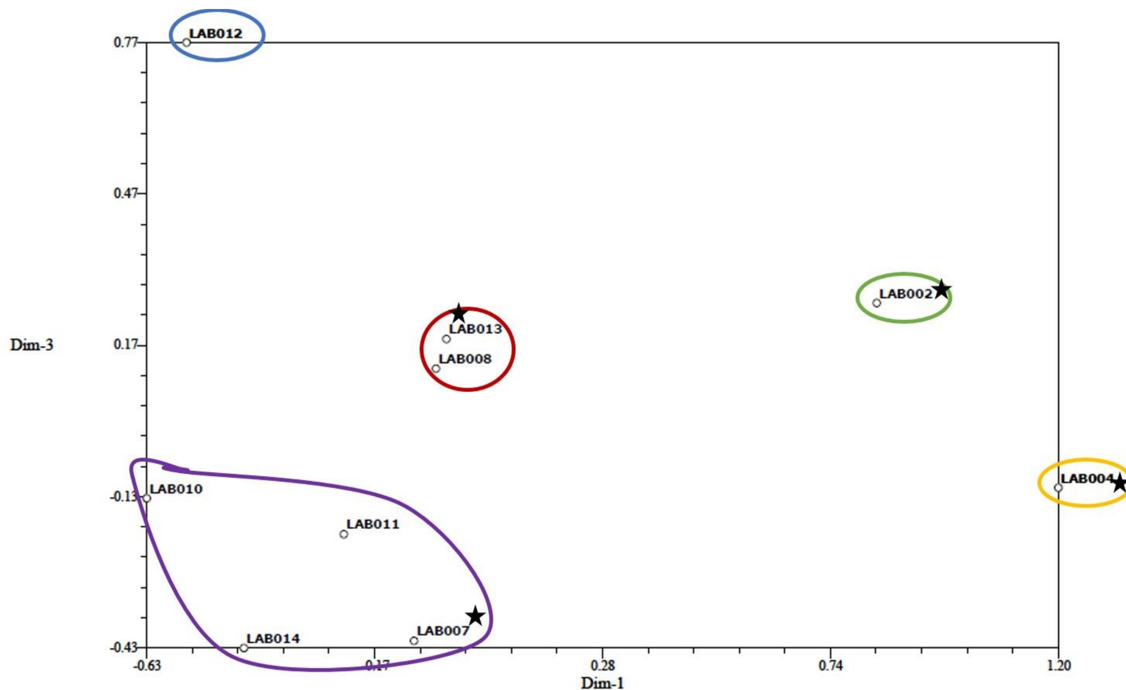

Figure 7. PC1 x PC3 projection of PCA of strains isolated from spontaneous fermentation. Distribution on space of strains defined by PC1 (Dim-1) and PC3 (Dim-3). The matrix used in PCA was 45 by 9 (45 conditions by 9 strains), being the variation associated to PC1 of 38.96% and to PC3 of 14.22%. Each circle correspond to a defined group of strains. Stars represent the strains selected for controlled fermentations.

The major group is constituted by four strains of three genera and four distinct species, being the similarity due to their absence of growth in different conditions such as higher concentrations of salt (higher than 6 %) in both 25ºC and 28ºC temperatures, and in three distinct pH analysed (4.5, 5.5, and 6.5).

The other group formed by more than one strain is constituted by *Lactobacillus plantarum* LAB 013 and *Lactococcus lactis* LAB 008. This group was formed since these strains growth in the same conditions as in every temperatures (25ºC, 28ºC and 30ºC) with lower concentrations of NaCl (0 and 3%), and essentially in pH more near of neutral (6.5).

In relation to other three groups each one composed by only one strain, these are distinguished of the other two groups by the global pattern in conditions of growth. In space defined by PC 1 and PC 2 the group of LAB 002 and LAB 004 have a higher distance essentially by PC 2, maybe by the fact that LAB 004 strain present a higher growth in low temperatures (25ºC) and LAB 002 in 28ºC, although both in higher pH. In the other way, both strains are proximal by PC1. Although the relation by this PC is not clear, there is an evidence that both strains grow more in lower pH in either temperatures of 25ºC and 28ºC and the other three groups grow worst in the same conditions. Thus, in pH 4.5 at 0% of NaCl and at 25ºC, LAB 002 grew 31.6% and LAB 004 30.4%, while other strains do not present any growth, with exception of LAB 007.

This analysis also showed that strains of same species did not group, thus highlighting the high intra-specific variability of lactic acid bacteria that was verified by the differences in ability to grow in different conditions and in growth rate of strains of the same species, in some cases.

3.5. Analytic determinations in culture medium

An important characteristic of microorganisms used as starter cultures is the ability to produce organic acids, in this case lactic acid, since it contributes to inhibition of spoilage agents [Hitendra, *et al.* 2016].

So, after incubation during 12 h at 28ºC, production of lactic acid and consumption of glucose were determined during the growth curve of each strain analysed, over 11, 12 or 13 h depending on the strain. This assay was required to select the strains with the better characteristics to be used as starter cultures in further controlled fermentations.

Some strains only produced one type of isomer of lactic acid, so according to literature, the enzymatic tests only were performed if the strain produces this type of lactic acid. Thus strains that produce only D-lactic acid were subjected only to a kit that detects D-lactic acid and the strains that produce L-lactic acid were subjected to a kit for L-lactic acid detection.

Initial glucose was calculated to each strain, to understand this sugar consumption. The strains that consume more glucose were both strains of *Leuconostoc citreum*, since all glucose of medium was consumed. In relation to production of lactic acid, these strains only produced D-lactic acid, producing a total of 11.2 g/L and 10.3 g/L in *Leuconostoc citreum* LAB 004 and *Leuconostoc citreum* LAB 011, respectively. The remaining glucose consumed can be used to biomass production and cellular maintenance. The strain that produced less lactic acid was *Lactobacillus hilgardii* LAB 010, with only 1.1 g/L of total lactic acid (L(+) and D(-)) produced, consuming only 4.8 g/L of glucose (Table 2).

The strains of genera *Lactococcus* and *Lactobacillus* (excluding *Lb. hilgardii* LAB 010) consumed approximately the same quantity of glucose but the production of lactic acid was the double in *Lb. plantarum*, comparing with *Lactococcus* and *Lb. brevis* strains, which means that *Lb. plantarum* are more appropriate for industrial applications.

Table 2. Sugar consumption and lactic acid production in the end of approximately 12 hours of growth in MRS at 28ºC aerobically. Results represent the means of the three replicates ± standard error.

Strains	Glucose consumption (g/L)	L-Lactic acid production (g/L)	D-Lactic acid production (g/L)	Total Lactic acid (g/L)
***Lactococcus lactis* LAB 002**	11.1±0.70	7.0±0.73	--------	7.0±0.73
***Lactococcus lactis* LAB008**	13.4±0.49	5.7±0.17	--------	5.7±0.17
***Leuconostoc citreum* LAB004**	17.9±0.00	--------	11.2±0.14	11.2±0.14
***Leuconostoc citreum* LAB 011**	18.2±0.00	--------	10.3±0.55	10.3±0.55
***Lactobacillus brevis* LAB 007**	10.9±0.61	3.1±0.41	3.3±0.17	6.5±0.59
***Lactobacillus hilgardii* LAB 010**	4.8±0.57	0.6±0.04	0.6±0.04	1.1±0.07
***Lactobacillus plantarum* LAB 012**	12.9±0.15	6.6±1.30	8.3±0.53	14.9±1.06
***Lactobacillus plantarum* LAB 013**	13.0±0.11	7.6±3.80	5.80±0.33	13.4±3.69

From these results, some strains were excluded to perform pepper fermentations at lab-scale. The strains selected presented the best yield, and when there was two strains of same species, only one was selected.

3.6. Pepper fermentation at lab-scale: towards a prototype

3.6.1. Starter cultures selected

After understanding the growth behaviour of each strain in MRS medium, only the strains with better ability to acidify the medium, producing lactic acid and tolerating different environments, with different pH, temperatures and concentrations of NaCl were selected.

So, only four strains were selected to be used in controlled pepper fermentations as starter cultures, *Lactococcus lactis* LAB 002*, Leuconostoc citreum* LAB 004*, Lactobacillus brevis* LAB 007 and *Lactobacillus plantarum* LAB 013. These strains were isolated from natural microbiota of different

peppers and the spontaneous pepper fermentation with 5% of NaCl and belong to well separated PCR fingerprintings clusters (see Figure 3).

Furthermore, observing the 2D plots of Principal Component Analysis (Figure 6 and 7) it is possible to see that from groups defined by growth in different conditions, one strain of each group was selected to be used in fermentations, with exception of *Lb. plantarum* LAB 012, since this strain belong to the same species of LAB 013.

3.6.2. Growth and acidification in different pepper pastes

With the objective of producing pepper pastes of quality, 16 pepper pastes were prepared, to evaluate the performance of each strain in acidification, growth capacity, sugar consumption and acid production.

The acidification of each strain was determined in two types of pepper pastes, sweet and hot, both with 5% and 0% of salt and allowed to analyse the capacity of strains to ferment different pepper pastes (Figure 8). The initial values of pH of the pepper paste were between 4.5 and 5.5 in every conditions, approximately to that indicated to pepper pastes (4.6 – 5.0) [Bozkurt and Erkmen, 2004].

There were greater differences between the control pepper paste (without starter culture) and the pepper pastes with selected strains, in relation to evolution of pH along fermentation time, showing that pastes with inoculated starters have a higher potential to a better preservation of these. On uninoculated pepper pastes there was a loss of viability during fermentation, with the presence of several filamentous fungi and yeasts. The control presented high values of pH, making the medium more alkaline with levels of pH ranging from 5.0 to 8.0, in both peppers. However, the more alkaline control were in hot peppers, wherein bell peppers had a pH more acidic. In both controls (types of pepper pastes), the paste without salt reached basic values more rapidly than with 5% of salt. This fact can be explained, since in the control were not inoculated any type of strain and the salt promote the residual microflora (not removed after pepper pastes preparation), decreasing the pH with higher facility. Contrarily, the pepper pastes with starter inoculum presented lower values of pH, leading to acidification of media.

Lactococcus lactis LAB 002 was the strain who showed lower potential in acidification of media, with exception in hot peppers without NaCl. This leads to an increase in presence of spoilage, since they don't promote an acidic media unfavourable to spoilage, since the production of acid lowers the pH, inhibiting the appearance of undesirable microorganisms [Breidt *et al.*, 2013]. No pepper paste with this strain inoculated was further used, since none presented the conditions required to subsequent sensorial analysis.

The strain with the best ability to acidify the paste environment was *Lactobacillus plantarum* LAB 013, which presented the lowest pH in every pepper pastes, reaching levels of pH of ca. 3.0 after 12 days of fermentation, which is in concordance with literature about similar vegetables like fermented cucumber, with a final pH of 3.1 – 3.5 [Montet *et al.*, 2014]. This strain is normally responsible for the final phase of fermentation, so they are adapted to low pH and the results revealed that the first two days the pH keeps

similar to control with this strain, while a strain responsible for the first stages of fermentation like *Leuconostoc citreum*, decrease rapidly the pH initially [Breidt *et al.*, 2013].

There was a difference also in pastes with different types of peppers, being the sweet pepper pastes affected with less spoilage that makes sense since they presented a lower pH. Thus, in relation to sweet pepper pastes, every pastes were subsequently used, with exception of the pepper paste inoculated with *Lactococcus lactis* LAB 002 (with 0 and 5% of NaCl). In hot pepper pastes only pastes with strain *Lactobacillus brevis* LAB 007 (both concentrations of salt) and paste inoculated with *Lactobacillus plantarum* LAB 013 (only the paste without NaCl) were used in subsequent analysis. Although there is no information comparing different pepper pastes (hot and sweet), the fact the strains present in some hot pepper pastes don't inhibit the spoilage can be explained not only by pH but also by no adaption to capsinoids affecting their growth and acidity activity.

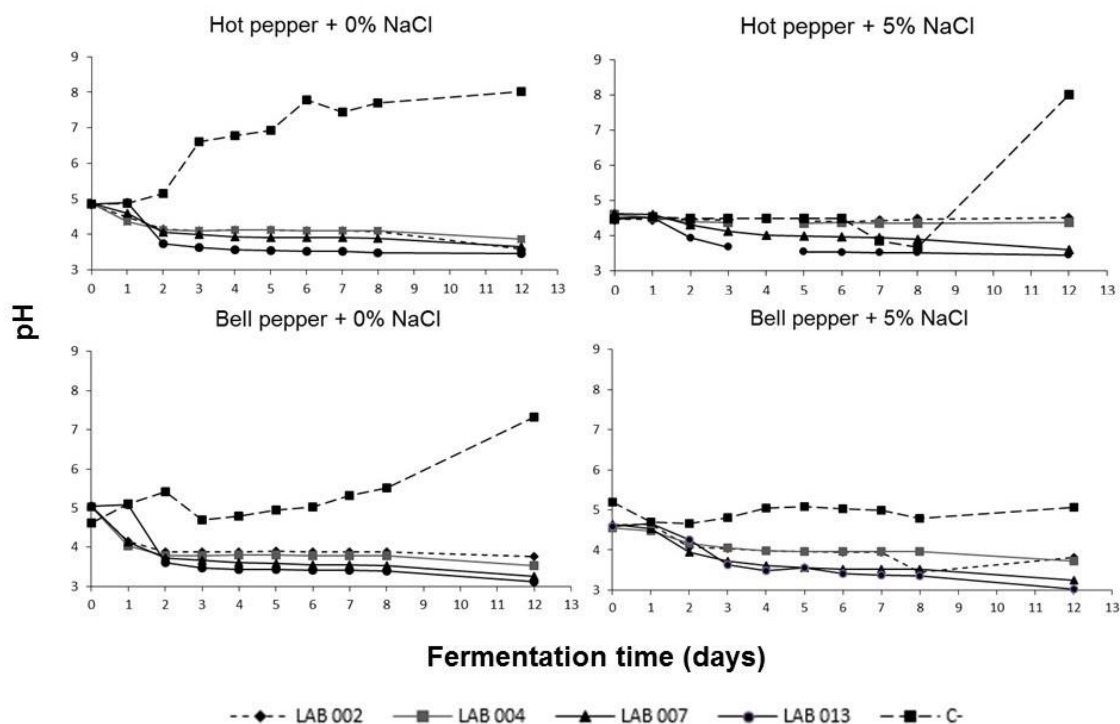

Figure 8. pH evolution during fermentation in different pepper pastes using different strains. The strains examined were *Lactococcus lactis* LAB 002, *Leuconostoc citreum* LAB 004, *Lactobacillus brevis* LAB 007, *Lactobacillus plantarum* LAB 013 and the control situation (C-). pH was measured every day, in a 12 days period, and the pepper pastes were incubated at 28ºC. In absence of point the pH was not measured.

In relation of kinetics of growth, isolates grew from 6.0 or 7.0 log CFU mL^{-1} to cell densities which ranged from 8.0 or 9.0 log CFU mL^{-1} in exponential phase. These values are concordant with the literature in fermentation of peppers, being this cell density frequently used in several vegetables [Di Cagno *et al.*, 2008]. In some pepper pastes there was a decrease in number of cells per mL during fermentation, reaching values of 5.0 log CFU mL^{-1} (Hot pepper pastes with 0% and 5% of NaCl both inoculated with

Leuconostoc citreum LAB 004 and hot pepper + 0% NaCl with *Lactococcus lactis* LAB 002), which is also concordant with literature since *Leuconostoc* spp. are predominant in the initial phase of the fermentation process of vegetables, but die when pH decrease [Breidt *et al.*, 2013]. Besides this, it was also possible to observe that in some pepper pastes the number of microorganisms present was constant, although this fact was not detected in any paste with *Lactobacillus plantarum* LAB 013, showing that this strain is a persistent strain.

3.6.3. Fermented pepper pastes

Sixteen pepper pastes were obtained after 12 days of fermentation at 28ºC in canning jars pre-sterilized. Four controls were also made to compare with pepper pastes inoculated with different strains.

Through figure 9A it is possible to observe that in pastes with bell peppers only two pastes are inappropriate for consumption, since they were target of spoilage contamination, which shows that antimicrobial activity of *Lactococcus lactis* LAB 002 is reduced. Since contaminated, the sweet pepper pastes with 0% and 5% of NaCl with this strain are inappropriate to use.

In relation to hot pepper pastes (Figure 9B), the maintenance of each strain in these pastes was not so effective, since only three pepper pastes were free of spoilage agents. The remaining suffered contamination. One more time the two conditions inoculated with *Lc. lactis* LAB 002 were contaminated as pastes with *Leuc. citreum* LAB 004 and the paste with 5% of salt inoculated with *Lb. plantarum* LAB 013. These results can show that some strains, which did not grow, are not adapted to saline environments.

The controls in both types of pepper pastes presented growth of yeasts and fungi. However the presence of starter cultures in pastes seems to help to avoid the presence of such contaminants. These contribute to the preservation of the pepper pastes since they produce lactic acid and consequently lead to the decrease of pH. Additionally they might help by competing with other present microorganisms.

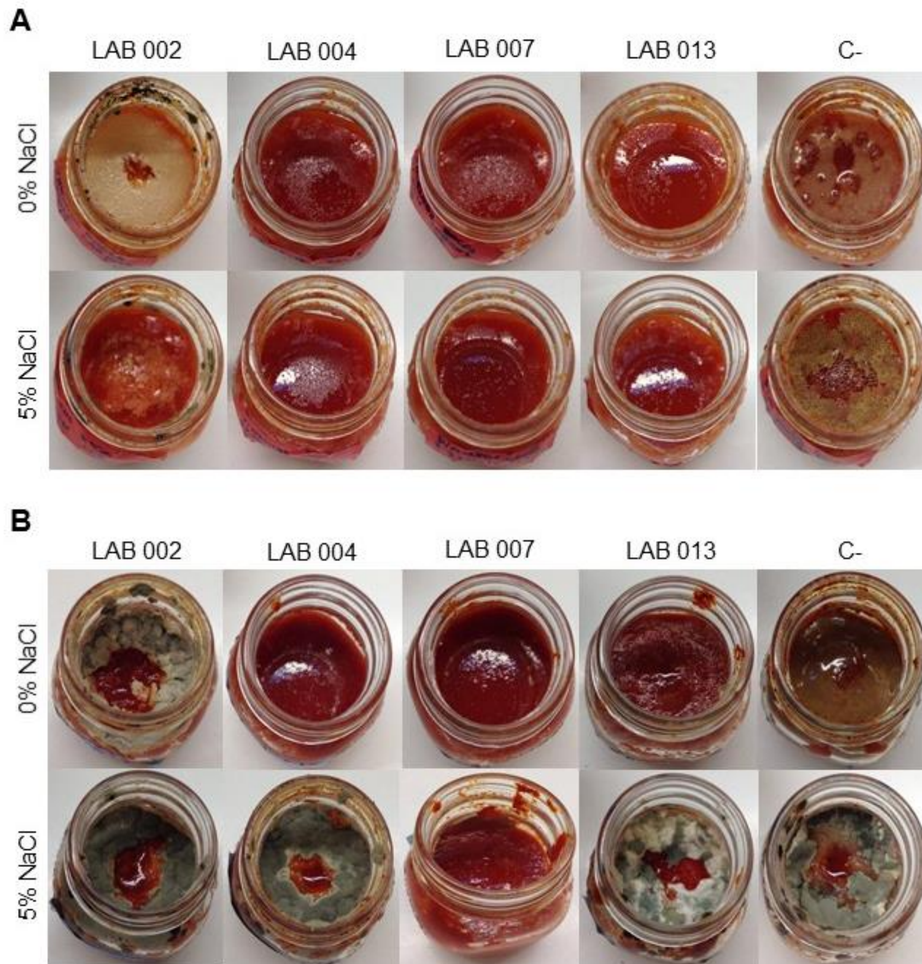

Figure 9. Pepper pastes inoculated with different strains and with different concentrations of NaCl (0 and 5%) obtained at the end of 12 days of fermentation. (A) Bell pepper pastes and (B) Hot pepper pastes.

3.6.4. Traceability of LAB starters during pepper fermentation

During pepper pastes fermentation there was a monitoring of growth through platting on MRS medium. This was required to control the microbiota present in fermentation and to assess if the starter culture remains in each pepper paste. The colonies with different morphology of starter cultures inoculated in each pepper paste were subjected to a Gram test and their morphological characterization was also realized.

The appearance of yeasts was perceptible from the second day in hot pepper pastes with 5% of salt and from the third day in hot peppers without salt, both inoculated with *Lactococus lactis* LAB 002. Yeasts were also present in sweet pepper pastes with this strain, showing one more time that LAB 002 does not have the characteristics needed to be use as a starter culture. Relatively to pastes inoculated with *Leuconostoc citreum* LAB 004 only the hot pepper pastes had yeasts. From remaining pepper pastes only were isolated coccus and rods. Some in pairs, in chains, or without aggrupation. Relatively to controls, in every negative controls were found yeasts, fungi and cocci and rods gram positive, which were in chains or isolated. Rods in filaments were also characterized.

Besides of negative control, in other pepper pastes were also isolated fungi from hot and sweet pastes with *Lactococcus lactis* LAB 002. *Penicillium* was the genus present in several pastes, with 6 isolates found. Also *Cladosporium* strains were present in pepper pastes.

On the other way, strains with similar morphologic characteristics to the inoculated strains were isolated and submitted to M13 PCR fingerprinting, to compare with fingerprints of initial starter cultures. A dendrogram for each pepper paste was performed to control the microbiota present in different phases of each fermentation (Figures 10, 11, 12 and 13). In each dendrogram set it is possible to see the different strains isolated during each fermentation where the ref. name (0xxTx_xY) is composed by an alphanumeric code constituted by three numbers that correspond to the inoculated strain, Tx that refers to the time of fermentation, the last x correspond to number of isolate and the Y to the type of fermentation (B- Bell pepper + 0% NaCl, V- Bell pepper + 5% NaCl, A- Hot pepper + 0% NaCl and L- Hot pepper + 5% NaCl). The level of reproducibility of this fingerprinting method is around 95%, so above this level is not possible to say that these strains are different.

Relatively to pepper pastes inoculated with *Lactococcus lactis* LAB 002 (Figure 10) it is possible to observe that this strain couldn't keep up during fermentations, except in bell peppers with 5% of NaCl where it is possible to see that this strain LAB 002 remained until T2. The fact of this strain does not remain in different pepper pastes can be related with the appearance of yeasts and fungi in these pepper pastes, since this strain was not present to guarantee the inhibition of potential spoilage. This species has not been associated with vegetable fermentation, but sometimes it is isolated in small number, being mainly found in the first stages of fermentation of some fermentable vegetables, which points to its inability to persist [Harris *et al.*, 1992].

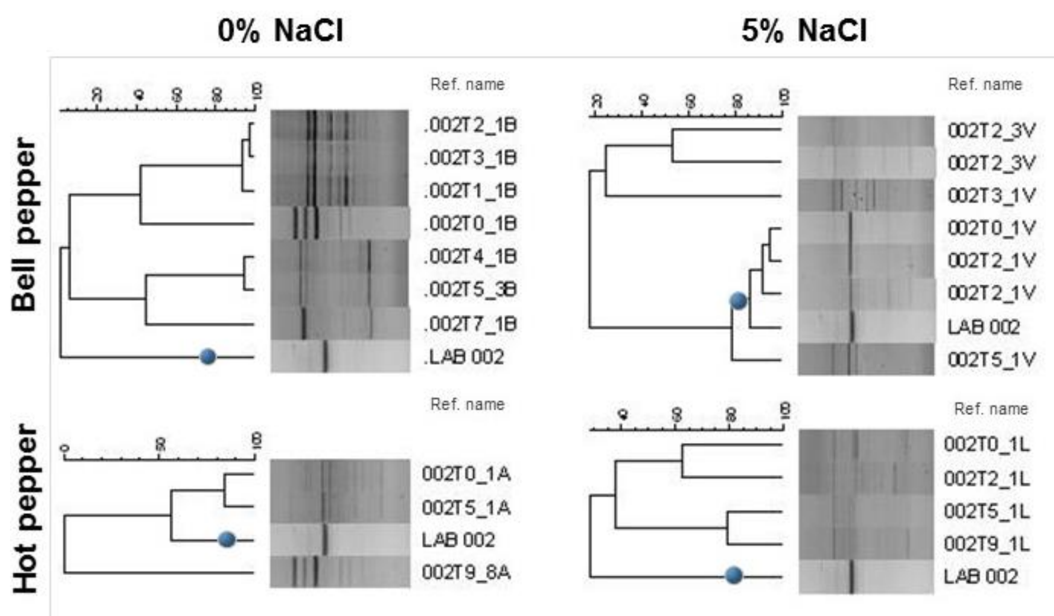

Figure 10. Traceability of *Lactococcus lactis* LAB 002 in different pepper pastes. The blue circle represents the cluster of strain *Lc. lactis* LAB 002.

In pepper pastes with *Leuconostoc citreum* LAB 004 (Figure 11), the starter culture keep up in every conditions, but in hot peppers with salt this only can persist until third day of fermentation. However, in this pepper paste in the first two days, this strain was not isolated. One more time, in the fermentation where the strain does not remain, the growth of yeasts was verified.

Furthermore, in the hot pepper paste without salt, although the inoculated strain persisted until the final phase of fermentation, there was the appearance of indigenous bacteria, which can be related with the weak action of this strain in acidification of this pepper paste.

In the other two bell pepper pastes, the cluster of *Leuc. citreum* LAB 004 includes isolates in the final phase of fermentation with confidence about 95%, being related with the capacity of strain to confer quality to pepper pastes.

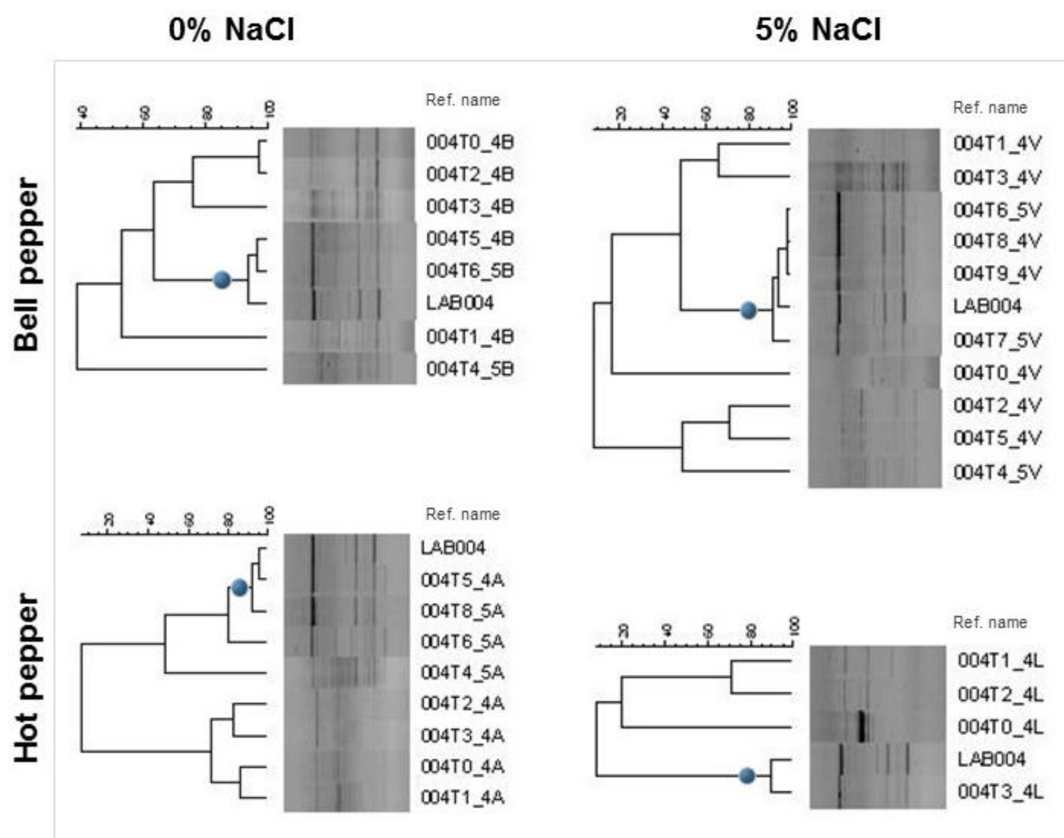

Figure 11. Traceability of *Leuconostoc citreum* LAB 004 in different pepper pastes. The blue circle represents the cluster of strain *Leuc. citreum* LAB004.

Relatively to pepper paste inoculated with the starter culture *Lactobacillus brevis* LAB 007 (Figure 12), this strain was not isolated during fermentation, since this appear alone in a distinct cluster in almost every conditions. The pepper paste with bell peppers and 0% of salt is the only where it's possible to observe a cluster of identical isolates. However there are differences between isolates and the starter culture in end of fermentation, so the strain not persisted until the final phase of fermentation. Since these pepper pastes do not exhibit spoilage, the absence of isolation of the starter can be explained by

the fact that the indigenous strain(s) in pepper paste persisted during fermentation, inhibiting pathogens and contributing to sensorial characteristics of pepper pastes, or by a failure of sampling of colonies of the starter in the final phase of fermentation.

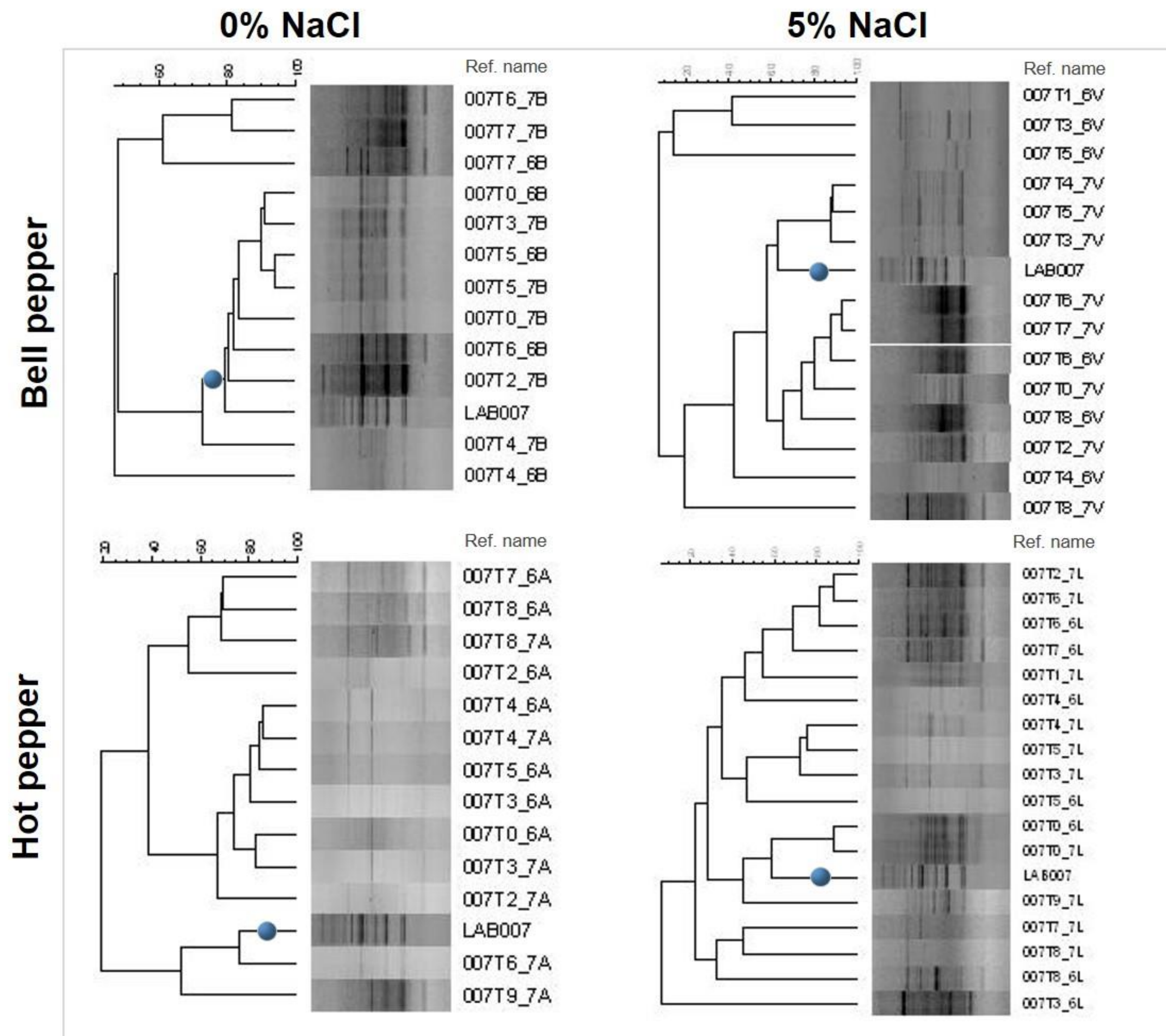

Figure 12. Traceability of *Lactobacillus brevis* LAB007 in different pepper pastes. The blue circle represents the cluster of strain *Lb. brevis* LAB007.

In the last set of fermentations with *Lactobacillus plantarum* LAB 013, a cluster of strains (Figure 13) obtained over fermentation is much defined, with exception in hot pepper fermentation + 5% of NaCl, where the inoculated strain occurs alone. Thus, according to other data previously analysed, *Lb. plantarum* LAB 013 showed to be a strain with the best capability to be used in pepper pastes, since being present, the appearance of spoilage does not happen. So, only in pepper paste where the strain has not remained, contamination exists (hot pepper fermentation + 5% of NaCl). In paste with bell peppers + 5% NaCl it is possible to consider two clusters of this strain, depending if we are more or less conservative (blue circle or green circle, respectively). So, we can assume that the salt has a notable influence on the participation of this strain in hot peppers.

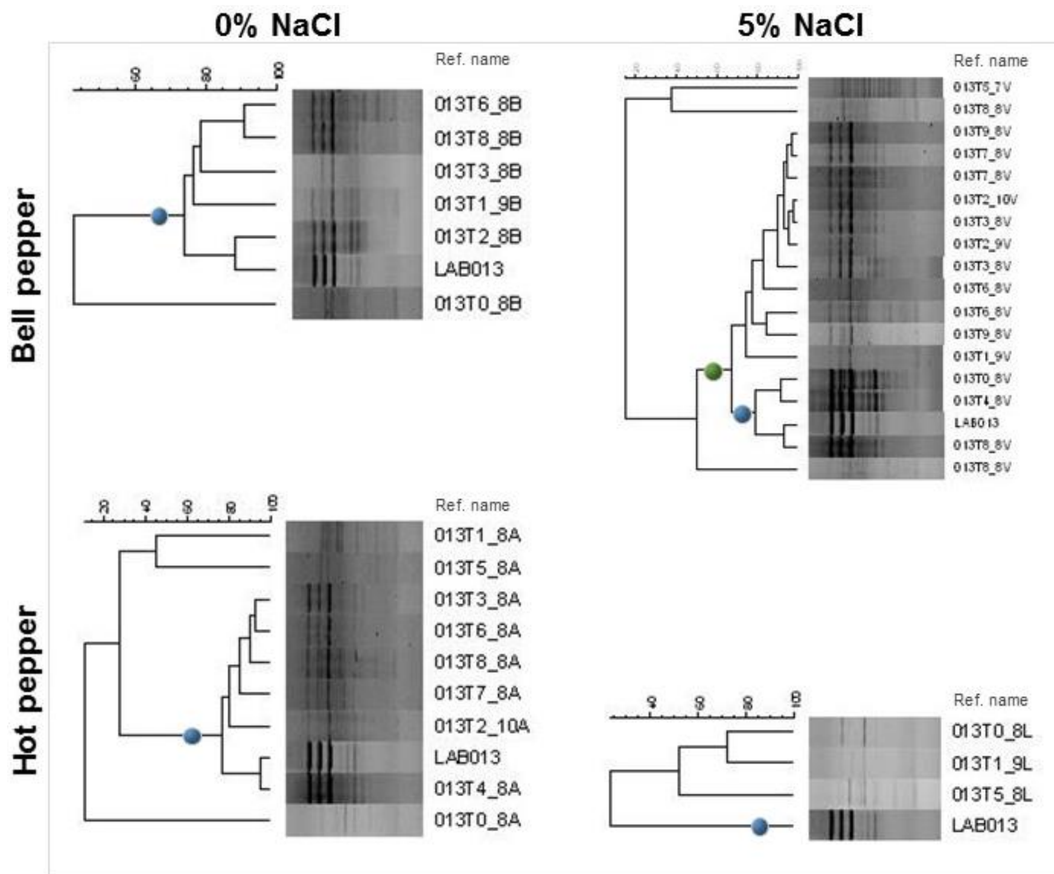

Figure 13. Traceability of *Lactobacillus plantarum* LAB 013 in different pepper pastes. The blue circle represents the cluster of strain *Lb. plantarum* LAB 013 and the green circle represents the hypothetic cluster of this strain, being less conservative.

3.6.5. Consumption of sugars in pepper pastes

Pepper pastes were analysed for the concentration of consumed sugars (glucose and fructose) over fermentation. Thus, during the fermentation were analysed the supernatant of three points, one in initial phase, other in exponential phase and other in stationary phase (points 0, 5 and 12).

Both pepper pastes contained a higher concentration of fructose than glucose, with concentrations of 34.1 g/L of fructose and 23.2 g/L of glucose in hot peppers and 33.7 g/L and 23.8 g/L of fructose and glucose, respectively, in bell peppers (Figure 14).

In all pepper pastes was observed that there was a higher consumption of glucose in pastes without salt. Relatively to fructose, it was also consumed in higher quantity in every pastes with 0% of NaCl.

Lactobacillus brevis LAB 007 was the strain that presented the highest consumption of sugars, in every pepper pastes, being all sugars practically consumed in hot pepper and bell peppers pastes without salt.

On the other way *Lactobacillus plantarum* LAB 013 presented the lowest consumption of fructose in pastes of bell peppers + 0% of NaCl, which makes sense since they are found in the final stages of fermentation where the most of sugars have already been consumed [Breidt *et al.*, 2013]. According to

literature [Alberto *et al.*, 2013], assays with *Lb. plantarum* as starter culture in pepper fermentations with 4% of salt and 20 g/L of initial glucose gave a total of 15,2 g/L of glucose consumed, while in this experimental the glucose consumed was only of 9,1 g/L in bell peppers with 5% of NaCl. Values similar to the referred in literature were achieved only with 0% of salt. Also pepper pastes with *Lb. plantarum* had an increase of sugars concentration in the end of fermentation, which can be explained by diffusion of sugars from the vegetables to the brine medium [Gardner *et al.*, 2001].

Finally, *Leuconostoc citreum* LAB 004 showed a lower use of both sugars in bell peppers with 5% of salt, and also a low concentration of glucose in bell peppers without salt.

3.6.6. Lactic acid production in pepper pastes

Lactic acid was also measured at three points for fermentation time, point 0, 5 and 12 h (Figure 14). Depending on the strain, only one type of lactic acid, or the two types were evaluated. Only *Leuc. citreum* produces one type of lactic acid, in this case the D-lactic acid, the other two strains used in this experiment produce both types.

Lactic acid production was different depending on the strain inoculated in each pepper paste. *Lb. plantarum* LAB 013 was the strain that presented the best production of total acid in both types of peppers, bell peppers and hot peppers and *Leuc. citreum* LAB 004 presented the lowest production of acid in pepper pastes analysed, that are related with higher resistance to acid of facultative heterofermentative LAB comparing with obligate heterofermentative strains (*Leuc. citreum*) [Breidt *et al.*, 2013].

Considering the conditions of pepper pastes, in paste without salt there was a higher production of lactic acid for every strains, in both types of peppers. For the paste inoculated with *Leuc. citreum* LAB 004 the production of D-lactic acid was 2.5 times higher in bell peppers without salt than in peppers with 5% of NaCl. In relation to pastes with *Lb. brevis* LAB 007 the production of total lactic acid was 1.8 times higher in hot peppers without salt than in hot peppers with NaCl and in bell peppers with this strain 2.3 times. At the last, in bell pepper pastes with *Lb. plantarum* LAB 013 the production of acid was 1.6 times higher in paste with 0% of NaCl than in with NaCl. This shows that strains are better adapted to conditions without salt, since they produce more acid when the salt is not present. Furthermore, *Lb. plantarum* LAB 013 is the strain better adapted to saline environments, because it presented a lower difference in production of lactic acid between pastes than the other strains. This fact can be related with the presence of strains of this species in brined vegetables with high concentrations of NaCl (above 5%) in pickled cucumbers and olives [Breidt *et al.*, 2013].

Relatively to types of peppers, comparing pepper pastes inoculated with same strain, the hot pepper pastes obtained a higher acid production, i.e. the environment in this type of peppers is more suitable for these strains. However this was not sufficient for a maintenance of more strains relatively to in bell pepper pastes.

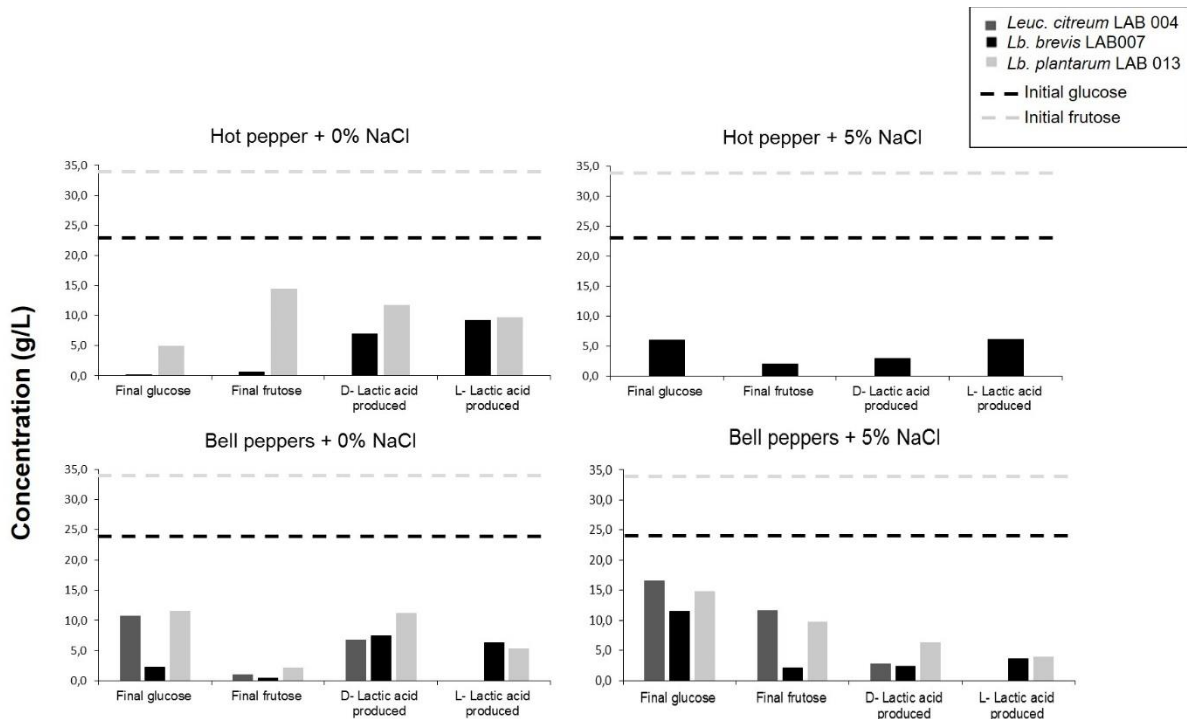

Figure 14. Sugar consumption and lactic acid (D- and L-) production during fermentation of hot and bell pepper pastes (0% and 5% of NaCl) with starter cultures. In absence of bar dosing was not performing. Results are means of triplicates.

In bell pepper pastes + 0% of NaCl the production of lactic acid was 13.8 and 16.5 g/L in pastes inoculated with *Lactobacillus brevis* LAB 007 and *Lactobacillus plantarum* LAB 013, respectively, which was similar to the values obtained in other studies of peppers fermentations (15.0±0.3 g/L) [Di Cagno *et al.*, 2009; Alberto *et al.*, 2013].

3.6.7. Sensory analysis – Characteristics of fermented pepper pastes

Sensory evaluation of peppers fermented under different conditions revealed that those fermented with *Lactobacillus brevis* LAB 007 in presence of salt in both bell and hot peppers pastes and the hot pepper paste without salt inoculated with *Lactobacillus plantarum* LAB 013 were considered the best. This conclusion was obtained by a question made in every questionnaires that allowed to deduce what is the best hot and sweet pepper pastes.

In relation to a discriminate sensory analysis, six characteristics were taken into account such as, aspect, colour, odour, flavour, residual flavour and acidity of different pepper pastes, which obtained a punctuation from 1 to 9, based on the taste of each one. The response of each characteristic was illustrated with radar charts in Figure 15.

Every pepper pastes inoculated with *Lactobacillus plantarum* LAB 013 were the pastes that presented the higher area of superficies in different categories, with exception of hot peppers with 5% of NaCl, where there isn't term of comparison in different strains. *Lactobacillus brevis* LAB 007 was the strain

with the best capability to colonize different types of pepper paste, since it persisted in every pastes. Nevertheless, strain *Lactobacillus plantarum* LAB 013 was the strain with the best characteristics.

The difference between pastes are small, so there is no highlighting of any pepper paste. Since *Lb. plantarum* present better organoleptic characteristics, the strains of this species are largely used as starter cultures in vegetable fermentations, being their dominance in this fermented food due to their metabolic capacity to adapt to different environmental conditions such as acidic media [Di Cagno *et al.*, 2009].

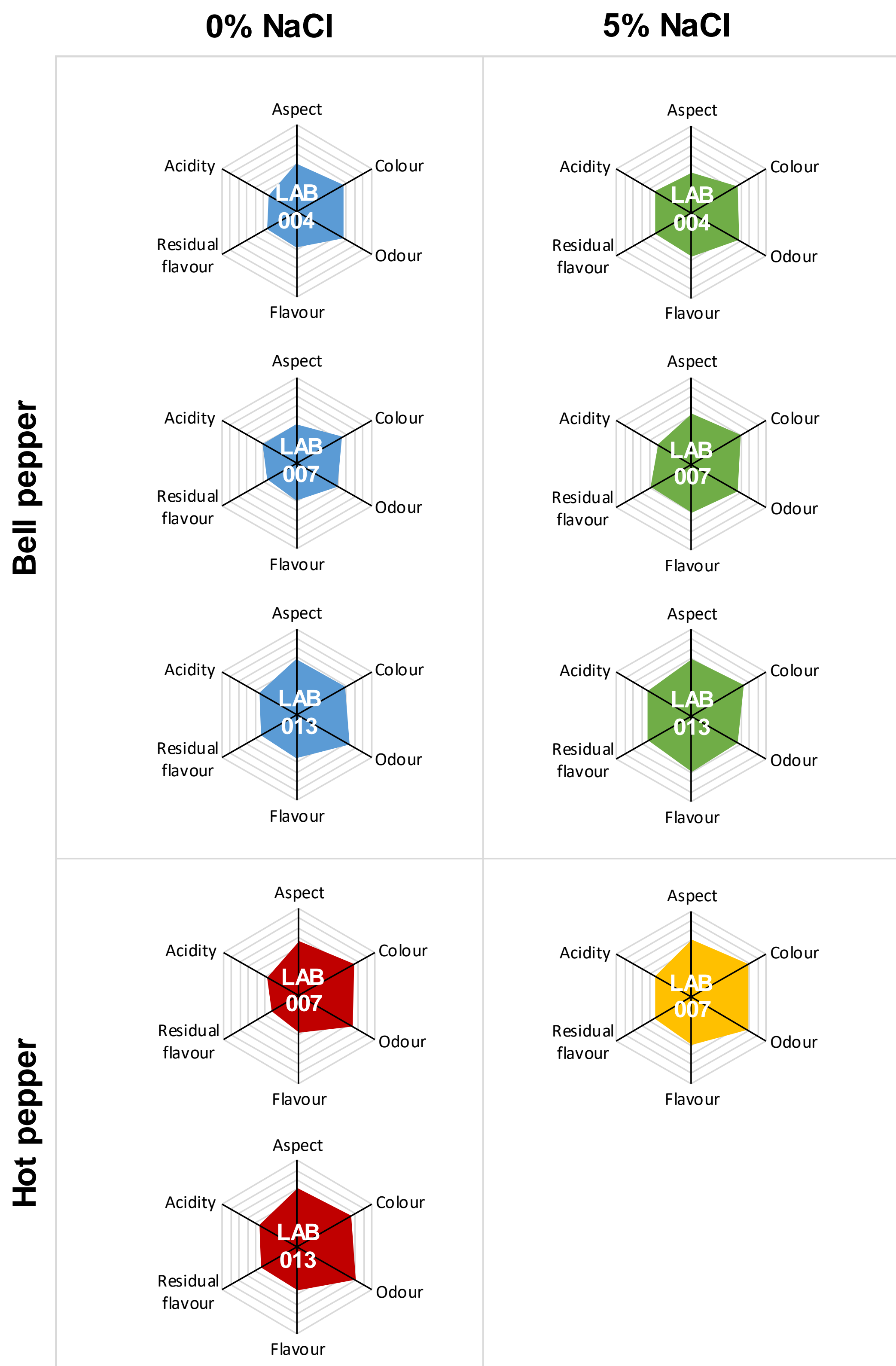

Figure 15. Illustrations of sensorial analysis of different pepper pastes of bell and hot peppers, with 0% and 5% of NaCl. Each value in different character is the average of evaluation given by 10 different tasters. The axes varies between 1 and 9.

In relation to yield of lactic acid in different pepper paste, the yield of one of selected pepper pastes characterized as the best (bell pepper + 0% NaCl with *Lactobacillus plantarum* LAB 013) was the highest amongst all pepper pastes. So, there is a relation between the concentration of lactic acid and the quality of pepper paste. In relation to pH over fermentation, was also *Lactobacillus plantarum* LAB 013 that obtained the lower pH (3.46) in the selected pepper paste. Furthermore *Lactobacillus brevis* LAB 007 also presented very low values of pH in both pepper pastes selected (3.24) in bell peppers + 5% NaCl and 3,61 in hot peppers + 5% NaCl).

4. GLOBAL OVERVIEW AND CONCLUSIONS

Pepper paste is a traditional fermented product obtained from mashed peppers belonging to the *Capsicum annum* species [Di Cagno *et al*., 2009].

Nowadays this type of product is widely used due to their organoleptic characteristics as flavour, colour, and aroma, and also for their probiotic activity, properties that are obtained by spontaneous growth of microorganisms of natural microbiota of peppers – Lactic acid bacteria. In the last years, the fermented food consumption has got special attention, but little information is available about fermentation of peppers.

Besides the lactic acid bacteria (LAB), the microbiota of peppers is also constituted by a large number of spoilage and sometimes pathogen microorganisms, making the microbial activity of LAB important as a traditional bio-preservation method and for extension of shelf-life of products [Di Cagno *et al*., 2009].

In the current study, from 80 strains with LAB characteristics, strains of species *Enterococcus gilvus*, *Enterococcus casseliflavus*, *Enterococcus faecalis*, *Enterococcus gallinarum*, *Enterococcus mundtii*, *Lactococcus lactis*, *Lactobacillus brevis*, *Lactobacillus plantarum*, *Weissella cibaria*, *Leuconostoc citreum* were identified. Overall, the species isolated and identified in pepper pastes were also found in other vegetables matrices and in some fermented vegetables [Andersson *et al*., 1988]. *Lactobacillus plantarum* and *Lactobacillus brevis* were identified in the microbiota of cucumber, tomato, eggplant, kimchi, sauerkraut and pickles. Besides peppers *Weissella* spp. occur in tomatoes and papaya. *Leuconostoc* spp. are present in cucumbers and carrots as well as in peppers [Di Cagno *et al*., 2009; Di Cagno *et al*., 2013]. In relation to *Lactococcus* spp., these species had not been described in fermentation of vegetables, although they were found in fermentation of Almagro eggplants [Seseña and Palop, 2007].

One important characteristic of LAB in fermentation processes is the development of acidity on environment, to avoid the presence of yeasts or fungi and to contribute to better characteristics in sensorial evaluation [Breidt *et al*., 2013]. As such the pH was measured in both MRS and in fermented pepper pastes environment, to disclose the most adapted strains to be used as starter cultures. So it was verified that during growth curves and in every fermented pepper pastes, *Lb. plantarum* LAB 013 was the strain that presented a better capability in acidification of media, which may be related to its ability to adapt to acid stress [Di Cagno *et al*., 2009] and its activity on the last phase of fermentation [Breidt *et al*, 2013]. This fact leads to the exploitation of this strain as a potential starter culture in several experiments of fermentation of peppers.

The quick decrease on pH is conducted by the production of organic acids, which are obtained by the conversion of sugars into lactic acid. So the concentration of these compounds were also measured and *Lb. plantarum* LAB 013 showed to be the strain with a higher production of lactic acid.

To cope with the view of Di Cagno *et al.* (2008), the selection of starter cultures to use in fermented vegetables involved the characterization of 1) growth rate, 2) production of organic acid, with consequent decreased of pH and 3) environment adaptation.

The characterization of each strain isolated from different types of peppers was translated into a diversity of strains, where four strains of 3 genera were used in different pepper pastes with distinct concentrations of NaCl to obtain a sample of each one. As described in Alberto *et al.* (2013), the pastes with 0% of salt favoured the microbial activity, being observable a lower decrease on pH in this pastes. So it may be conclude that salt limits the pH decrease and sometimes bacterial growth, since the permanence of inoculated strains is not verified. However, two of the pastes selected as the best used 5% of salt being both inoculated with *Lb. brevis*, which also was described in Seseña *et al.* (2001) in eggplants fermentation as important, since this obligate heterofermentor may avoid development of an unpleasant taste. Furthermore, also the paste with *Lb. plantarum* was considered the best hot pepper paste (0% NaCl).

So, for their abilities to promote a better set of organoleptic characteristics, and to persist in several types of pepper pastes, *Lb. plantarum* LAB 013 and *Lb. brevis* LAB 007, respectively, were the most promising strains for starter cultures. Studies with Almagro eggplants showed that the use of these starter cultures at the same pepper paste revealed to be the best option, by inoculating *Lb. plantarum* and *Lb. brevis*, since they promote good sensorial panel and a shorter fermentation time, with a sequential fermentation, where obligate heterofermenters act at the initial stage with subsequently action of facultative heterofermenter [Seseña *et al.*, 2001].

In this work the occurrence of interactions between microorganisms in pepper fermentation is evident. The selected LAB strains demonstrated to have an inhibitory effect in potential spoilage agents present in different pepper pastes, which is observable comparing pepper pastes with and without inoculated starter cultures.

Currently, the consumption demand of fruits and vegetables is referred to be lower than the recommended doses by the Food and Agricultural Organization, being the daily uptake of these suggested, since they are essential in health nutrition. To work around this problem, lactic acid fermentation can be a solution for increasing the daily consumption of vegetables [Montet *et al.*, 2014].

LAB perform an essential role since they are responsible for the majority of fermented food, such as cucumbers, peppers, olives and other vegetables. With the use of LAB and consequent lactic acid obtained from these bacteria, it is possible to achieve fermented vegetables, in this case pepper pastes with organoleptic characteristics of quality without the addition of any chemical additives, prejudicial to health. LAB starter cultures have been increasingly used to obtain reproducible and improved quality of fermented vegetables. These bacteria can acidify the media, avoiding the deterioration of products, with the presence of spoilage agents, since LAB compete with other microorganisms for nutrients and habitat, eliminating those [Alberto *et al.*, 2013]. Thus, the main goal of this work was to obtain starter cultures from natural microbiota of peppers to use in further fermentations, to enhance health and the sensorial properties of peppers.

5. FUTURE PERSPECTIVES

Lactic acid bacteria are a group of microorganisms largely employed at industrial level, being used in a variety ways like food manufacture, health improvement and production of macromolecules and enzymes [Giraffa, 2014].

Although the use of fermentation with these bacteria is an ancient process, in last decades their application in food won special attention. In an era where the 'safe' products are fashionable, the industrial food market has grown with fermented food performed with these bacteria. However, the world of fermented food is not completely explored. For this is required to discover and characterize new strains of LAB, where the metabolism of these bacteria is the key word to use subsequently as starter cultures. These new strains can be obtained either by the same way that 'old' strains (from raw material and spontaneous fermented foods) or using genetic engineering [Giraffa, 2014].

The present work intended to produce new innovative products at the market level, through the conception of pepper pastes fermented by a well-defined and characterized bank of starter cultures. In the future, a metagenomic approach, using next generation sequencing (NGS), could be useful to understand the diversity of the LAB strains present in each pepper. This methodology will allow either the sequencing of conserved regions, such as the 16S rRNA gene, or whole genome sequencing. With the first approach it is possible to understand the diversity associated with each sample while the latter allows the search for gene-based functions in the community, which can positively affect the organoleptic characteristics of pepper pastes (e.g. production of diacetyl). Additionally, it would help to unveil the necessary requirements for the growth of these interesting strains in a NGS-assisted isolation.

Relatively to controlled fermented processes, in the future could be interesting to use mixed cultures to inoculate the same pepper paste, since each type of bacteria is responsible for a stage of fermentation. In order to avoid the contamination with spoilage other techniques as pasteurization can be applied. Yet, this can be prejudicial since this heat treatment can reduce the quality of the pepper paste, affecting the colour and the consistence of this product [Alberto *et al.*, 2013].

This project also aims to develop an optimized industrial production process of fermented pepper pastes, using up-to-date techniques from research labs that are little addressed at industrial level. This unusual practice can create new and different products with quality and revolutionize the food industry.

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