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BIOACTIVITY OF HIGHLY HYDROLYTIC BACTERIA ISOLATED FROM CHIAPAS CHEESE (MEXICO)

by

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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Applied Microbiology

by

Joana Filipa Horta Machado

Thesis performed in the *Research facilities of the Department of Food and Nutritional Sciences, University of Reading, United Kingdom*

Under the supervison of

Prof. Dr. Paula Jauregi

&

Under the co-supervison of

Prof. Dr. Manuela Pintado

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Dedicated to my parents,
who always picked me up on time and encouraged me to go on every adventure,
especially this one.

Resumo

As bactérias do ácido láctico (BAL) são amplamente utilizadas na indústria alimentar. Este grupo de microrganismos não só têm a capacidade de produzir ácido láctico, que contribui para o sabor e a textura dos produtos fermentados, mas também possuem a capacidade de produzir, durante a fermentação do leite, péptidos bioactivos que podem ser importantes para a saúde humana por terem actividade anti-hipertensiva, antimicrobiana, antioxidante ou anti trombótica. O queijo creme de Chiapas, queijo tradicional mexicano, é obtido através de uma coagulação ácida e enzimática prolongada de leite cru de vaca e tem características sensoriais únicas devido às BAL endógenas no leite desta região. Assim, o objectivo deste projecto foi seleccionar e estudar a bioactividade de isolados altamente hidrolíticos do queijo Chiapas. Uma selecção inicial de 84 isolados foi realizada para produção de diacetil e uma produção negativa de catalase. Uma análise adicional para medir o grau de hidrólise e para obter o perfil de péptidos foi realizada. Os isolados seleccionados foram comparados com *Lactobacillus helveticus* DSM 13137 e utilizados para estudar a actividade potencial dos péptidos libertados durante a fermentação láctica. As propriedades antioxidantes de fracções de soro de leite, após 24h e 48h de fermentação em leite reconstituído desnatado (LRD), foram avaliadas por dois métodos espectrofotométricos. Através da captura do radical 2,2´- azinobis (3-etilbenzotiazolina-6-ácido sulfônico) (ABTS) variou entre 50%-86%, enquanto que pelo ensaio do poder antioxidante redutor do ferro apresentou valores de FRAP variáveis entre as amostras seleccionadas. Um ensaio de inibição da enzima de conversão da angiotensina (ACE) por HPLC foi realizado, mostrando inibições médias de 75% nos isolados em comparação com 96% em DSM 13137. Os efeitos inibidores potenciais dos péptidos bioactivos produzidos por os isolados seleccionados também foram testados contra alguns organismos patogénicos: *Escherichia coli*, *Salmonella* Typhi, *Salmonella* Enteritidis e *Listeria monocytogenes*, usando o método de disco de papel e mostrando casos com zonas de inibição de diâmetro de 12 mm, em média. Os resultados indicados sugerem que estas estirpes podem ter aplicações como starters na indústria de lacticínios e como fonte de novos péptidos bioactivos com diferentes modos de acção.

Palavras-chave: Bactérias Lácticas, queijo Chiapas, péptidos bioactivos, fermentação.

Abstract

Lactic acid bacteria (LAB) are widely used in food industry. This group of microorganisms not only have the ability to produce lactic acid, which contributes to the taste and texture of fermented products but also show the capability to produce, during milk fermentation, bioactive peptides that can be important in human health by having antihypertensive, antimicrobial, antioxidative, antithrombotic and mineral-binding activities. Chiapas cream cheese, a traditional type of Mexican cheese, is obtained through a prolonged acid-enzymatic coagulation of cow raw milk and has unique sensory characteristics due to the endogenous LAB in milk from this region. Thus, the aim of this project was to select and study the bioactivity of high hydrolytic isolates from Chiapas cheese. An initial selection from 84 isolates was made for production of diacetyl and a negative production of catalase. Further analysis to measure the degree of hydrolysis and to obtain the peptide profile was carried out. Selected isolates were compared to *Lactobacillus helveticus* DSM 13137 and used to study the potential activity of peptides released through lactic fermentation. The antioxidant properties of whey fractions, after 24h and 48h of fermentation in reconstituted skimmed milk (RSM), were evaluated by two spectrophotometric methods. Scavenging of the cation radical of 2, 2'-Azino-Bis (3–Ethylbenzthiazoline–6-Sulfonic Acid) (ABTS) ranged from 50%-86% while ferric reducing antioxidant power assay presented variable FRAP values among the selected strains. An angiotensin-converting enzyme (ACE) inhibitory assay by HPLC was performed showing inhibitions by average mean of 75% in the isolates compared to 96% in DSM 13137. The potential inhibitory effects of the bioactive peptides produced by the selected isolates were also tested against some food-poisoning pathogenic organisms: *Escherichia coli*, *Salmonella* Typhi, *Salmonella* Enteritidis and *Listeria monocytogenes*, using the paper disc method, showing cases with inhibitory zones by average mean of 12 mm diameter zone. The results indicated suggest that these strains could have applications as starters in dairy industry and as a source of new bioactive peptides with different modes of action.

Keywords: Lactic Acid Bacteria, Chiapas cheese, bioactive peptide, fermentation.

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To everyone, muito obrigada!

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List of Abbreviations

AA	Antioxidant activity
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ACE	Angiotensin-converting enzyme
BHI	Brain heart infusion
°C	degree Celsius
CFU	Colony-forming unit
DH	Degree of hydrolysis
DNA	Deoxyribonucleic acid
FDA	Food and Drug Administration
FRAP	Ferric reducing antioxidant assay
g	gram
h	hour (time)
HA	Hippuric acid
L	Litre
LAB	Lactic Acid Bacteria
M	molar
meq	milliequivalent
mg	milligram
µl	microlitre
mL	millilitre
µm	micrometer
MRS	de Man, Rogosa and Sharpe medium
OD	Optical density
PBS	phosphate-buffered saline
PCR	Polymerase chain reaction
RP-HPLC	Reversed Phase High Performance Liquid Chromatography
rpm	rotations per minute
RSM	Reconstituted skimmed milk
TEAC	Trolox equivalent antioxidant capacity
w/v	weight/volume

1. Introduction

Due to a growing demand for food with attractive organoleptic characteristics and microbiologically safe, the development of new products or improving existing ones has been a constant these days [1]. Lactic acid bacteria (LAB) have been widely used by food industries, not only as a biopreservative since is one of the oldest and highly efficient forms of non-thermal processing method but also to add distinct flavours and aromas to the end product [2, 3].

Cheese is one of the most significant products fermented by these microorganisms. In Mexico, cheese-making is one of the most important industries and it uses approximately 25% of the total milk produced in the country [4]. While many dairy products, if properly manufactured and stored, are biologically, biochemically and chemically very stable, cheese is, in contrast, biologically and biochemically active [5].

Artisanal cheeses are usually obtained through a prolonged acid-enzymatic coagulation of cow raw milk which has endogenous LAB, adding unique sensory characteristics that vary from region to region. Interestingly, it has been reported that some strains of LAB possess compelling health-promoting properties through the bioactive peptides they release during milk fermentation [6-10].

The traditional fermented products are complex ecosystems, often unknown, which constitute a source of new strains of LAB that could be potential sources of new genes encoding variants of bacteriocins, proteases and / or other enzymes [5].

1.2. Chiapas cream cheese

Chiapas cream cheese is a traditional ripened cheese from the southern Chiapas state in Mexico. It is known for its sour taste and relatively high salt content (5.34%) [11]. During its manufacturing, it undergoes a stage of acidification, for several hours at room temperature, by native species of lactic acid bacteria, forming a fresh and soft paste. This type of cheese is made with cow's milk, raw or anger, in whole or partially skimmed, from one of the three different regions of Chiapas: North, Frailesca (center) and Costa (coast) [11,12].

This tropical cream cheese enjoys great prestige in the state of Chiapas and Tabasco where it is consumed, popularly accompanying the daily food, and has great potential for obtaining a PDO (protected designation of origin) [13].

Recently, scientists tried to characterize the endogenous LAB present in Chiapas cheese by molecular techniques, showing different dominant genera according to season and region [14].

However there are still limited studies about the potential bioactivity of peptides produced by endogenous LAB isolated from this type of cheese.

1.3. Lactic Acid Bacteria

Lactic acid bacteria are a wide range of phylogenetically related genera of Gram-positive (Figure 1), non-spore-forming bacteria, which produce lactic acid as the major end product of sugar fermentation [1, 15]. They are generally catalase and oxidase negative with a rods or cocci shape [1].Besides being aerotolerant anaerobes, organisms that tolerates the presence of oxygen but does not require it for growth, these microorganisms are mesophiles and have complex nutritional requirements especially for amino acid and vitamins [15]. Therefore, they require proteolytic systems capable of hydrolysing proteins into peptides or amino acids. For example, the Lactococcal proteolytic system consists of enzymes outside the cytoplasmic membrane, transport systems, and intracellular peptidases [16].

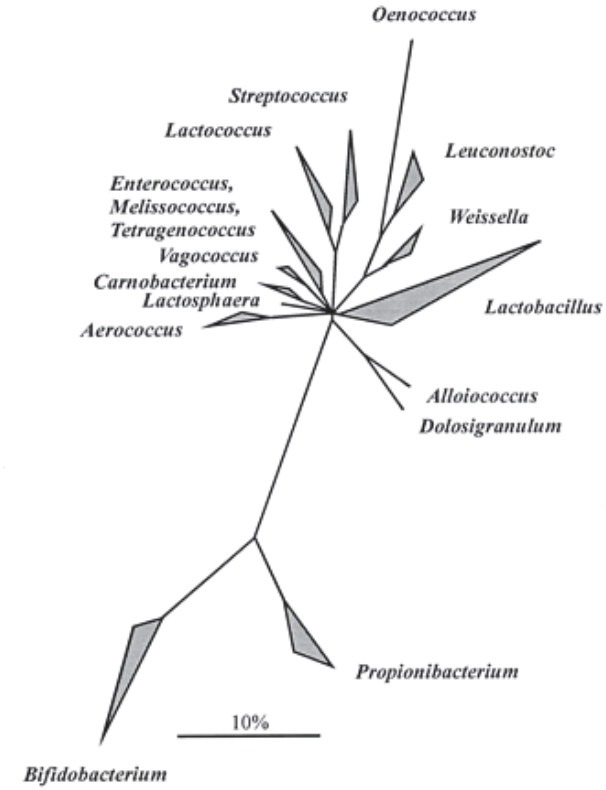

Figure 1. Concensus tree, based on comparative sequence analysis of 16S rRNA, showing major phylogenetic groups of lactic acid bacteria with low mol% guanine plus cytosine in the DNA and the nonrelated Gram-positive genera Bifidobacterium and Propionibacterium.
Adapted from Schleifer & Ludwig (1995) [17].

Based on their sugar fermentation patterns, is possible to divide this diverse group in two categories: homofermentative (or homolactic) bacteria such as Lactococcus and Streptococcus,

which follows the Embden-Meyerhof pathway and results in almost exclusively lactic acid as the end product; and heterofermentative (or heterolactic) bacteria, for example Leuconostoc and Weissella, which follows the 6-phosphogluconate/phosphoketolase pathway and results in other end products such as ethanol, acetic acid and carbon dioxide in addition to lactic acid [1, 3, 15].

This characteristic is not only useful for their taxonomy, but also in the food industry since heterolactics are more relevant in producing flavour and aroma components such as acetylaldehyde and diacetyl, than the homolactics [16].

1.3.1. Applications in food industry

Lactic acid bacteria are widespread in nature and are among the most important groups of microorganisms used in food fermentation [3]. Although LAB are comprised of 11 genera, only 6 of them are dairy associated: Lactococcus, Enterococcus, Streptococcus, Leuconostoc, Pediococcus, and Lactobacillus [18, 19]. They are involved in making yogurt, cheese, cultured butter, beverages, soy sauce, sour cream, and many more [1]. These bacteria not only contribute to the development of the desired sensory properties in the final product but are also capable of inhibiting food spoilage, increasing its microbiological safety and shelf life [2]. It has also been reported the use of LAB to control mould growth as an alternative to the existing physical and chemical methods [20].

These abilities can be achieved through two ways: fermentation products, where the antimicrobial activity can be exerted through the reduction of pH or production of organic acids (lactic acid, acetic acid), CO_2, reuterin, diacetyl, 2-pyrorelidone, 5-carboxylic acid (PCA), preventing the pathogen and contaminant growth that may occur during cheese making process; or through bacteriocins, which can be defined as protein antibiotics of relatively high molecular weight and mainly affecting the same or closely related species [21-23].

It is known that LAB are generally regarded as safe microorganisms and so are their bacteriocins [22]. Nisin was the first bacteriocin known and used for many years in numerous countries as a food additive to extend the shelf-life or to control specific microorganisms such as *Clostridium* spp, *Listeria monocytogenes* and other Gram-positive pathogens [24, 25].

Lactobacillus helveticus is traditionally used in the manufacture of Italian cheeses and it is the prevalent species recovered from natural lactic starter cultures [26]. These microorganisms help enhance the flavour of the cheeses and can help reduce or prevent bitterness. They are also gaining importance as health-promoting culture in probiotic and nutraceutical food products [27]. It has the potential to produce bioactive peptides or bacteriocins, and exert symbiotic effect when associated with prebiotics in fermented dairy products [26, 27]. *Lactobacillus*

helveticus can therefore be considered as a multifunctional LAB with increasing importance in the food industry [27].

1.3.2. Probiotic effects in human health

LAB are regarded as a major group of probiotic bacteria [28]. The probiotic concept has been defined by Fuller (1989) to mean “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance” [29]. Lactic acid bacteria have been shown to have a positive impact on human health when consumed as viable cells (probiotic). Relevant literature reviews have been written with an emphasis on health roles for lactobacilli and bifidobacteria in humans and/or animals [29, 30, 31]. These microorganisms colonize the intestine and are reported to have different effects. They produce substances inhibitory to pathogenic microorganisms, reduce the cholesterol level in the blood serum of pigs and alleviate lactose intolerance in V galactosidase deficient persons [32, 33, 34]. They also have been shown to have an anti-cancer effect, to stimulate the immune system and lowering blood pressure [35].

The blood pressure is mediated through Angiotensin Converting Enzyme (ACE) which plays a key role in its regulation. This enzyme is found in the rennin angiotensin system and have been reported by several researchers that fermented milk with probiotics LAB can exert ACE inhibitory effect [36]. A few commercial functional dairy products enriched with ACE-inhibitory peptides such as Evolus® (Valio Ltd. Valio, Finland), Calpis sour milk marketed as Ameal S® (Calpis Food Industry Co., Ltd., Tokyo, Japan) and Casein DP-Peptio® (Kanebo Co., Ltd., Kanebo, Japan) have been introduced as additional or alternative treatments for hypertension. The antihypertensive effects of these milk have been tested in vivo using hypertensive human subjects and spontaneously hypertensive rat models [37, 38, 39].

For these reasons consumption of fermented dairy products has been advocated by some physicians, scientists and non-scientists. In fact, lactobacilli are now sold in pharmacies and health food stores under a variety of names and to treat a number of illnesses [40].

2. Aims of the study

The present project aims at selecting high hydrolytic lactic acid bacteria isolated from Chiapas cream cheese and the study of peptides activity released during milk fermentation. The specific objectives of this work were:

- To screen all the isolates for biochemical properties and proteolytic performance;

- To investigate the potential activity of the peptides released by selected proteolytic strains, with a focus on antioxidant properties, angiotensin-converting enzyme inhibitors and antimicrobial activity.

3. Material & Methods

3.1. Biological material

For the present study, previously isolated colonies (here designated test bacteria) numbered from 1-87 were used. These isolates, listed in Table 1, were obtained from two samples of Chiapas cheese represented in Figure 2, through random and selective media based on the morphology and Gram staining of lactic acid bacteria. Bacterial isolates were then stored at -70ºC in cryovials (Microbank Pro-Lab Diagnostics, South Wirral, England).

Table 1. Codification of isolated colonies stored.

# code	Cheese	Medium	Atmosphere
1-6	Tabasco	MRS	Aerobic
7-18	Veracruz	MRS	Aerobic
19-28	Tabasco	MRS	Aerobic
29-35	Tabasco	MRS	Anaerobic
36-42	Tabasco	MRS	Anaerobic
43-47	Tabasco	Blood agar	Anaerobic
48-54	Tabasco	Blood agar	Anaerobic
55-60	Veracruz	MRS	Anaerobic
61-70	Veracruz	MRS	Anaerobic
71-77	Veracruz	Blood agar	Anaerobic
79-87	Veracruz	Blood agar	Anaerobic

Figure 2. Origin of the isolates used in this study: Double cream Chiapas cheese from Veracruz (left) and Double cream Chiapas cheese from Tabasco (right).

The potential bioactivity of the selected colonies was assessed against six target bacteria: *Escherichia coli* K12, *Salmonella enterica* serovar Typhi, *Salmonella enterica* serovar Enteritidis, *Listeria monocytogenes* 10403S, *Listeria monocytogenes* EGD-e and *Listeria monocytogenes* L028.

Lactobacillus helveticus DSM 13137 was chosen as a comparative reference strain since it is predominantly used in the fermentation of milk and has been reported to produce antihypertensive peptides [26, 27].

3.2. Media

Different media containing all the nutrients necessary for the growth of the isolates as well as the target organisms were used to perform the assays (Table 2).

Table 2. Media composition used in this study.

Identification	Composition (g/L)
De Man, Rogosa, Sharpe agar (MRSA)	10g of peptone, 8g of Lab-Lemco powder, 4g of yeast extract, 20g of glucose, 1mL of sorbitan monooleate, 2g of dipotassium hydrogen phosphate, 5g of sodium acetate $3H_2O$, 2g of triammonium citrate, 0.2g of magnesium sulphate $7H_2O$, 0.05g of manganese sulphate $4H_2O$, 10g of agar
Brain heart infusion broth (BHI)	12.5g of brain infusion solids, 5g of beef heart infusion solids, 10g of protease peptone, 5g of sodium chloride, 2g of glucose, 2.5g of disodium hydrogen phosphate
Brain heart infusion agar (BHIA)	12.5g of brain infusion solids, 5g of beef heart infusion solids, 10g of protease peptone, 5g of sodium chloride, 2g of glucose, 2.5g of disodium hydrogen phosphate, 10g of agar
Reconstituted skimmed milk (RSM)	100g of skim milk powder for microbiology (Sigma ®)

All media, except RSM, were sterilized by autoclaving at 121°C for 15 minutes and stored at 5°C. The reconstituted skimmed milk was pasteurized in a steamer for 20 minutes at 100°C and used on the same day.

3.3. Initial selection of the isolates

3.3.1. Activation of strains

For activation, the anaerobic strains were streaked with disposable sterile loops on MRSA (Oxoid ®, Basingstoke, Hampshire, England) and grown under anaerobic conditions: a mixture of CO_2, H_2 and N_2 in a ratio 10:10:80 respectively, in an anaerobic cabinet (Don Whitley Scientific Ltd Shipley, West Yorkshire, UK) for 48 h at 37 ºC. The aerotolerant strains were also streaked on MRSA and grown in an incubator for 48h at 30ºC.

3.3.2. Milk fermentation and sampling

The milk fermentations were carried out in hungate tubes (16x125mm) with 15mL of 10% (w/v) RSM. To each tube, an inoculum of 1% of a fresh overnight culture in BHI of the testing strain was added, in order to reach an initial number of 10^6-10^7 cells/mL, except in the negative control that only contained the pre-culture medium. The fermentations were performed in duplicates and incubated at 30°C under continuous agitation for 24h.

After the fermentation, the pH of each tube was measured using a pH-meter SevenEasy (Mettler-Toledo AG 8603, Switzerland) to ensure acidification has occurred. Samples of 4mL of each replicate were taken to determine the diacetyl production, total amount of proteins, degree of hydrolysis (DH) and peptide profile. These samples were heated up at 72°C for 1 minute to stop the enzymatic proteolysis and then centrifuged at 13000 rpm for 10 minutes. The supernatant was recovered, filtered through 0.45µm sterile filter (Minisart High-Flow, Sartorius, Germany) and stored at -25°C for further analysis.

3.3.3. Catalase test

With a sterile disposable loop, a small amount of a well isolated, 18 to 24 hours old colony from MRSA was transfered onto the surface of a clean microscope slide. Two drops of 3% hydrogen peroxide were added onto the smear. A positive result was a rapid evolution of O_2 by bubbling, while no bubbles was considered a negative result.

3.3.4. Diacetyl test

Diacetyl production was determined according to King (1948) as followed: 1 mL of milk previously inoculated with strains cultures (1%, v/v, incubation for 24 h at 30 °C) was mixed with 0.5 mL of a α-naphthol (1%, w/v in ethanol), followed by 0.5 mL of KOH (16% w/v) and incubated at 30 °C for 10 minutes [41]. Diacetyl production was indicated by a development of pink colour in the tubes, while no change of colour was considered as negative.

3.3.5. Total amount of proteins and degree of hydrolysis

The total amount of proteins in each sample was determined using bicinchoninic acid (Sigma-Aldrich). Twenty parts of bicinchoninic acid solution were mixed with one part of 4% (w/v) copper (II) sulfate pentahydrate solution before the analysis. Two millilitres of the reagent solution were added to 0.1mL of milk sample (soluble fraction) and incubated at 37°C for 30 minutes. Serial dilutions of bovine serum albumin (Sigma-Aldrich) were used as standard and the blank consisted only of buffer with no protein. The absorbance was read at 562nm within 10 minutes of each other using a spectrophotometer Ultrospec 1100 Pro UV/Vis (Amersham, Uppsala, Sweden). This assay was performed in duplicate. The standard curve was used to calculate the final protein concentration in mg/mL, multiplied by any dilution factor that was required to make in order to bring the concentration within the linear range of the assay.

The degree of hydrolysis (DH) was determined using the method of o-phthaldialdehyde (OPA) described by Nielsen *et al.* (2001) with some modifications [42]. Samples were previously diluted in deionised water to reach a concentration of 0.1-1mg/mL. 200µL of diluted fermented milk sample were added to 1.5 mL of OPA reagent (Sigma-Aldrich) in an assay tube and after 2 minutes incubation the absorbance was immediately read, using 2mL acrylic cuvettes, at 340nm in a spectrophotometer (Ultrospec 1100 Pro UV/Vis, Amersham). Deionised water was used as

a blank and a serine dilution (0.9516 meqv/L) was used as standard. The DH was calculated using the following equation (3.3.5.1):

$$DH\% = \frac{h}{htot} \times 100 \qquad \textbf{(3.3.5.1)}$$

Where DH% is the percentage of degree of hydrolysis and *h*, the number of hydrolysed bonds, is given in the equation below (3.3.5.2):

$$h = \frac{(Serine-NH2-\beta)}{\alpha\ meqv.g^{-1}} \qquad \textbf{(3.3.5.2)}$$

where α, β and h_{tot} with the values 0.4, 1 and 8.8 respectively, are constants for whey described by Nielsen *et al*. (2001) [38]. h_{tot} is the total number of peptide bonds per protein equivalent.

In order to calculate Serine-NH_2 the next formula (3.3.5.3) was used:

$$Serine - NH2 = \frac{(ODsample-ODblank)}{ODstandard-ODblank} \times 0.9516\ meqv.L^{-1} \times \frac{(S\times D)}{(P)} \qquad \textbf{(3.3.5.3)}$$

where Serine-NH_2= meqv.serine $NH_2.g^{-1}$ protein; S= sample volume (L); D= dilution factor; P= protein concentration of the sample (g/L). This assay was also carried out in duplicate.

3.3.6. Peptide Profile

The peptide profile was determined by RP-HPLC (reversed-phase high performance liquid chromatography) using a gradient described by Gonzalez *et al*. (2013) as followed: a mobile phase of solvent B (Trifluoroacetic acid 0.08% in acetonitrile) was run from 0 to 40% in 60 min, 40–70% in 5 min, 70–70% in 10 min, and 70–0% in 5 min, following a wash out of 100% of solvent A (Trifluoroacetic acid 0.1% in water) for 15 min [43]. The flow rate was 0.8 mL/min through a column (Ace 5 C18 250 × 4.6 mm 5 mm, 100 Å), reading at a wavelength of 214 nm. All the samples were filtered throughout sterile filters of 0.22µm (Ministart High-Flow) and injected two times into the Dionex HPLC system (Figure 3). This system consisted of an ASI-100 T automated sample injector, a photodiode array detector, PDA-100 and a computer with the Chromeleon v6.8 Sunnyvale datasystem software (all from Dionex Softron GmbH, Germany).

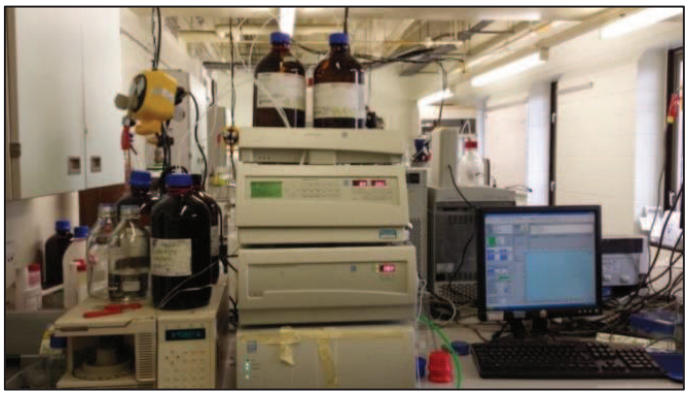

Figure 3. Dionex HPLC system used in this study.

3.4. Bioactivity assays

3.4.1. Milk fermentations and sampling

After the initial selection of the isolates, three strains were selected for the bioactivity studies. Milk fermentations were carried out in duplicate, in glass *Schotts*, with 100mL of 10% (w/v) reconstituted skimmed milk. An inoculum of 3% (v/v) of a fresh overnight culture in BHI of the selected strain as well as *Lactobacillus helveticus* DSM13137 was added, in order to reach an initial number of 10^6-10^7 cells/mL, except in the negative control that only contained the pre-culture medium. The glass bottles were incubated at 30°C under continuous agitation for 48 hours.

Samples of 5mL were taken after 0h, 24h and 48h of fermentation with sterile disposable graduated pipettes under aseptic conditions by the Bunsen burner. These samples were heated up at 72°C for 1 minute to stop the enzymatic proteolysis and then centrifuged at 13000rpm for 10 minutes. The supernatant was recovered, filtered through 0.45µm sterile filter (Minisart High-Flow, Germany) and stored at -25°C for further analysis.

3.4.2. Angiotensin-converting enzyme (ACE) inhibitory activity

The ACE-inhibitory activity percentage (ACEi%) was determined according to the method described by Gonzalez *et al.* (2011) [44]. It is a method based on the reaction of hydrolysis of N-Hippuryl-His-Leu (HHL) into hippuric acid (HA) and His-Leu (HL) catalysed by the angiotensin-converting enzyme (ACE). The activity of ACE was measured in terms of HA produced over time. 10µL of fermented milk sample was added to 30µL of ACE (60 mU, Sigma-Aldrich) and incubated at 37°C for 10 minutes, before 90 µL of 5mM HHL was added. After gently mixing it in a vortex it was incubated for 60 minutes at 37ºC. The reaction was ended using 8µL 5M HCl and the samples were filtered throughout sterile filters of 0.22µm (13mm, Kinesis®) into screw top vials (Conex chromatography systems, Scotland).

The concentration of HA produced at the end of the reaction was determined by reversed-phase high performance liquid chromatography (RP-HPLC) according to Mehanna and Dowling (1999) with some minor modifications [45]. A column Ace 5 C18 250×4.6 mm 5 mm, 100 Å (Advanced Chromatography Technologies, Scotland) was used in a Dionex HPLC system which consisted of an ASI-100 T automated sample injector, a photodiode array detector, PDA-100 and a computer with the Chromeleon v6.8 Sunnyvale datasystem software (all from Dionex Softron GmbH, Germany). A sample of 20 µL was injected into the system. The flow rate was 1 mL/min with an isocratic solution of acetonitrile 12.5% and trifluoroacetic acid 0.1% in water over 20 minutes and the area peaks corresponding to the HA concentration were obtained at 228nm absorbance. ACEi% assays were made by duplicate and each sample was injected two times into the HPLC system.

The ACEi% was calculated by subtracting the HA produced in the presence of the inhibitors to the HA produced in absence of inhibitors (under the same conditions) as shown below (3.4.2.1):

$$ACEi\% = \frac{[HAc]-[HAs]}{[HAc]} \times 100 \qquad (\mathbf{3.4.2.1})$$

where *HAc* is the concentration of hippuric acid produced by the ACE incubated in milk at 30°C for 0, 24 and 48h without the inoculum. This control was found to be more appropriate for this study than buffer as it reduced any interference from other components in the milk such as calcium that may induce inhibition [46]. *HAs* is the concentration of hippuric acid produced by the ACE in the presence of inhibitors contained in the fermented milk.

3.4.3. Ferric reducing antioxidant power (FRAP)

The FRAP assay is based on the reduction of Fe(III) to Fe(II) due to the action of antioxidants present. Subsequently, the Fe(II) formed may interact with 2,4,6-tris(2pyridyl)-s-triazine (TPTZ) providing a strong absorbance at 595nm [47].

This assay was performed according to Benzie & Strain (1996) with minor modifications [48]. FRAP reagent was prepared as a mixture of 2.5 mL of 10 mM TPTZ in 40 mM HCl and 2.5 mL of 20 mM $FeCl_3$ in 25 mL of 0.1 M acetate buffer (pH= 3.6). 10 µL of a fermented milk sample was mixed with 300µL of the FRAP reagent into a microcentrifuge tube. After gently mixing it in a vortex, 100µL were transferred in duplicate into a 96-well plate. Absorbance was measured immediately after in the plate reader (Tecan GENios) at 595nm.

Serial dilutions of ascorbic acid (Sigma-Aldrich) were used as standard and the calibration curve obtained (R^2 ≥0.99) was used for quantification.

3.4.4. Trolox equivalent antioxidant capacity (TEAC)

The trolox equivalent antioxidant capacity (TEAC) assay is based on the scavenging of the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical converting it into a colourless product. The degree of decolourization induced by a compound is related to that induced by trolox, giving the TEAC value [49].

In this assay, a solution of ABTS+ • radical was prepared by mixing 5mL of 7mM stock solution of ABTS with 88µL of 140mM potassium persulfate. The mixture was kept in the dark at room temperature for at least 16h prior to use. This solution was then diluted in phosphate buffer sulphate (PBS) until an absorbance of 0.700 ± 0.020 was reached at λ=734nm. 20µL of each milk fermented sample were added in Eppendorf tubes, in triplicate, followed by 2mL of the solution previously prepared. After 6 minutes in the dark, the mixture was transferred into a cuvette and the absorbance was read at λ=734nm in a spectrophotometer.

Trolox standards were used for quantification and data was expressed as μmol Trolox equivalent/L. The antioxidant activity (AA%) was calculated by the following equation (3.4.4.1):

$$AA\% = \frac{(ABScontrol - ABSsample)}{(ABScontrol)} \times 100 \qquad (\mathbf{3.4.4.1})$$

where *ABScontrol* is the absorbance of ABTS radical diluted in PBS and *ABSsample* is the absorbance of the reaction with the sample.

3.3.5. Antimicrobial assay

In order to obtain the cell-free extracts, each testing strain was cultivated in sterile flasks (20 mL) with 15 mL of BHI and incubated at 30°C under continuous agitation (200 rpm). After 24h, each culture was centrifuged for 3 minutes at 10000 rpm in order to get a supernatant which was then filtered through 0.22 μm filter (Ministart Highflow, Germany) and freezed at -25°C for future use. The pH values of the supernatants were neutralized to 6.8 by 0.1 NaOH to ensure that zones of inhibition were not the result of lactic acid production.

An overnight culture in BHI broth of the target organisms was also performed. Standardized inoculums (1x10^8 CFU/mL) were lawned onto sterile BHIA by swabbing evenly with sterile cotton swabs in three different directions. 6 mm sterile paper discs (Whatman, N3) were impregnated with 10μL of the supernatant and placed aseptically and distinctively onto the inoculated BHI agar plates (Figure 4), in duplicate. As a control, sterile BHI broth was absorbed in a disc and placed on BHIA plate. The agar plates were then incubated at 30ºC for 18h.

The sensitivities of the microorganisms to the supernatants were determined by measuring the sizes of inhibitory zones (including the diameter of disc) on the agar surface with a ruler.

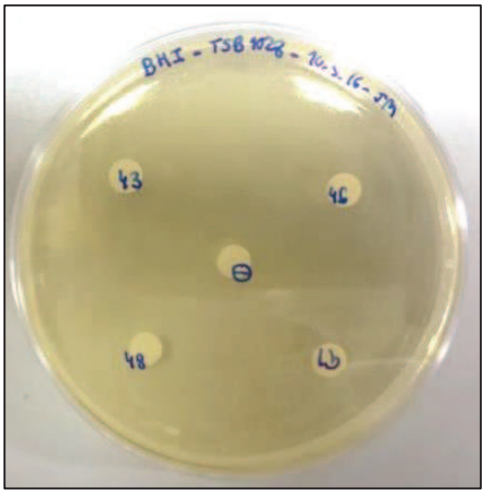

Figure 4. Representation of the disc placement method in the antimicrobial assay.

3.5. Molecular analysis

3.5.1. DNA extraction from pure cultures

In order to extract and purify the DNA of the isolates, a loopful of cells (from agar plates) was added to 0.5mL TES buffer. After mixing it with a vortex, 10μL lysozyme (10mg/mL) and 2μL mutanolysin (1mg/mL) were added to the cell suspension and incubated in a waterbath at 37°C. After 30 minutes, 10μL proteinase K (20mg/mL) and 10μL RNase (10mg/mL) were added to the cell suspension, separately, and the tube was incubated at 65°C for 1 hour. After incubation, 100μL of 10% SDS (sodium dodecyl sulfate) was added to the samples and incubated for 15 minutes more at the same temperature. The samples were then allowed to cool on ice for 30 minutes. Under a fume hood, 620μL phenol:chloroform:water was added and the contents of the tubes were gently mixed by inversion for 2 minutes. The samples were then centrifuged for 10 minutes at 6500 rpm. The aqueous (upper) layer was transferred to a clean and sterile microcentrifuge tube, using a wide-bore pipette tip. 1mL of ice-cold ethanol was added to the aqueous layer and after the contents were mixed, the samples were left on ice and stored overnight in a -20°C freezer. On the following day, the samples were centrifuged for 5 minutes at 13000 rpm and the supernatant was carefully removed. The tubes were inverted on a paper towel and allowed to dry for at least 3 hours. DNA was re-suspended in 50μL of sterile water and stored at -20°C.

DNA concentration and purity of each sample was then measured with a NanoDrop™ spectrophotometer and dilutions in order to obtain final concentrations of 5ng/μL were performed.

3.5.2. RAPD-PCR

Isolates were subjected to random amplified polymorphic DNA (RAPD) analysis in order to estimate their genetic variations.

The reaction mixture (25μL) consisted of 5μL GoTaq Flexi buffer (5x), 2.5μL dNTPs (0.4 mM of each of dATP, dCTP, dGTP, dTTP), 1.5μL $MgCl_2$, 1μL primer (20pmol/mL), 1μL GoTaq DNA polymerase (Promega), 1μL template DNA (5 ng/μL) and 13μL mili-Q water. The mixture was then incubated in the thermocycler (with heating lid) programmed for 40 cycles, each one consisting of a denaturation step (30 seconds at 94°C), one annealing step (60 seconds at 38°C) and an extension step (2 minutes at 72°C), an extra extension step was performed for 10 min at 72°C. The amplification was carried out with primer OPA-09 (5'-GGGTAACGCC-3'). The reaction products were separated by electrophoresis on an agarose gel (1.5%) containing ethidium bromide with final concentration of 0.5ng/mL prepared in 1X TAE buffer. A 1Kb DNA ladder was used as a molecular size indicator. DNA fragment (bands) patterns were then visualized under UV light (Genesnap, Syngene) and analysed by Gel Compar II software.

3.5.3. Bifidobacterium specific PCR

Although selective media for Lactobacillus was used in the bacterial isolation, a PCR with *Bifidobacterium* genus-specific primer sets Bif164-f (5'-GGGTGGTAATGCCGGATG-3') and Bif662-GC-r (5'-CGCCCGCCGCGCGCGGCGGGCGGGG-3') was performed.

The reaction mixture consisted of 5μL GoTaq Flexi buffer (5x), 2.5μL dNTPs mix, 1μL of each primer (both 20pmol/mL), 1μL GoTaq DNA polymerase (Promega), 0.5μL template DNA (5 ng/μL) and 14μL mili-Q water. The PCR thermocycling program with primers Bif164-f and Bif662-GC-r was the following: 94°C for 5 minutes; 35 cycles of 94°C for 30 seconds, 58°C for 20 seconds, 68°C for 40 seconds; and 68°C for 7 minutes. The reactions were subsequently cooled to 4°C. The size and amounts of PCR products were estimated by analysing 5μl samples by 2% agarose gel electrophoresis in 1x TAE buffer and ethidium bromide staining (0.5μg/mL). The bands were finally visualized under UV light (Genesnap, Syngene).

4. Results & Discussion

4.1. Initial selection of the isolates

An initial screening of 84 isolates from Chiapas cheese was performed for their ability to produce diacetyl and their absence of catalase activity. The catalase test has been used for many years for rapid differentiation of the genera of Gram-positive organisms and it is associated to pathogen species of Enterobacteriaceae [50]. The detection of diacetyl is an indicator of yeast or lactic acid bacteria presence [51]. Based on these two characteristics, a total of 24 isolates (19 from Tabasco and 5 from Veracruz cheese sample) were selected and used for further studies. A detailed board can be consulted in Appendix A (Page 25-26).

The total amount of proteins present in the fermentation samples of the selected isolates was then quantified using the bicinchoninic acid (BCA) method. The results showed concentrations by average mean between 3-12 mg/mL throughout the selected isolates, using bovine serum albumin (BSA) as a whey protein standard. This assay has a good combination of sensitivity and simplicity, being more accurate than other well-known protein determination methods such as Lowry or Bradford, especially due to the complex stability [52]. An accurate measurement of protein concentration was critical since the results were also used to determine the degree of hydrolysis.

Amino acids in protein molecules are linked together through amide bonds or peptide bonds [53]. During hydrolysis, these bonds are delinked or cleaved to produce peptides [54]. The percentage of the peptide bonds cleaved, or degree of hydrolysis, was determined based on a reaction between amino groups and OPA in the presence of DTT.

In Figure 5, seven isolates that revealed a similar or higher degree of hydrolysis than *Lactobacillus helveticus* DSM13137 (12.28%) are presented as well as the amount of protein measured in each sample.

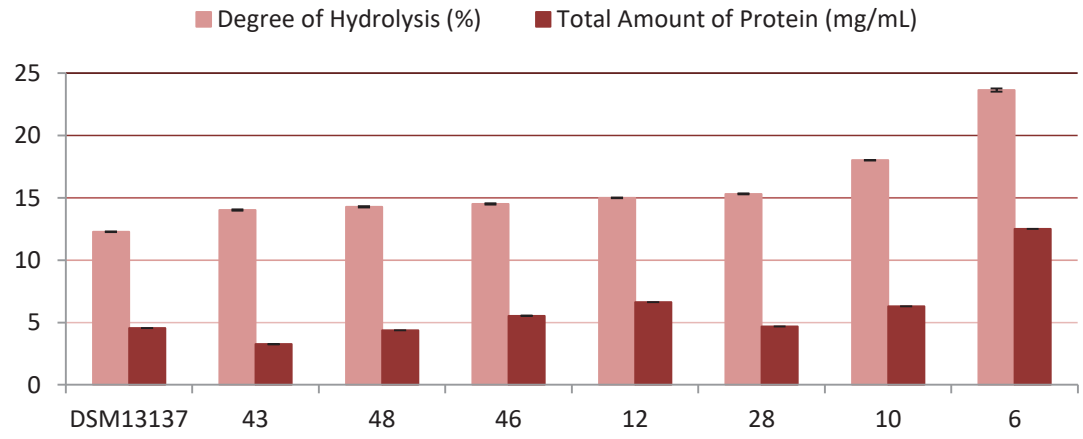

Figure 5. Degree of hydrolysis and total amount of protein present in samples of *Lactobacillus helveticus* DSM12137 and 7 unknown strains isolated from Chiapas cheese.

Although the degree of hydrolysis describes how much a protein substrate has been modified by an enzyme, a correlation between the total amount of protein and the hydrolysis degree is not absolutely necessary [42].

These results were confirmed by RP-HPLC analysis. The peptide profiles of the twenty four isolates were obtained, and the isolates with a higher degree of hydrolysis showed a higher number of peaks with different patterns while the samples with less than 5% DH almost showed any peaks, for example the strain 76 in Figure 6 did not reveal peptide release compared to strain 10 that have shown a degree of hydrolysis of 18% and is showing a significant amount of peptides after 24h of fermentation.

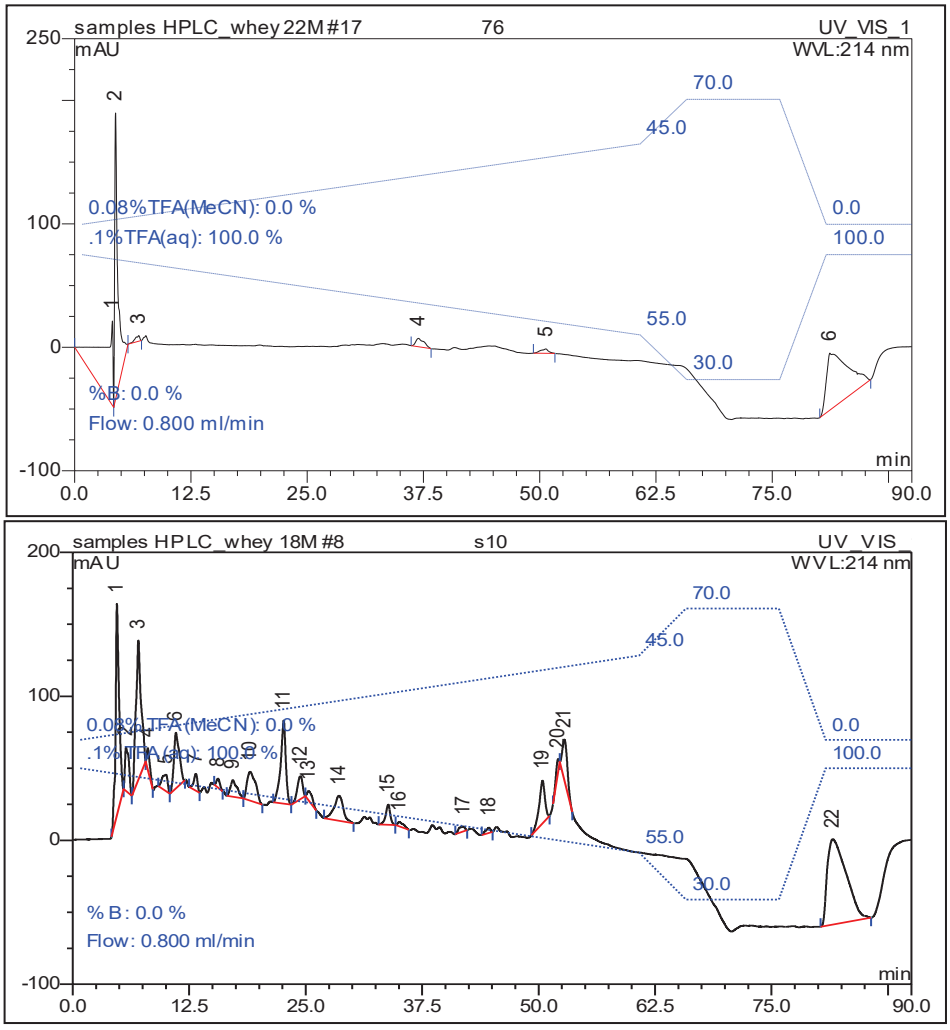

Figure 6. Comparison between degree of hydrolysis and peptide profile of sample 76 and 10.

From these results, the isolates 6, 10 and 12 showed a high degree of hydrolysis (>12.28 DH%), and therefore considered as highly hydrolytic isolates were selected to perform the bioactivity studies.

4.2. Bioactivity studies

4.2.1. ACE inhibitory activity

After a new set of fermentations, extracts of fermented samples from the chosen isolates were screened for ACE inhibition, as presented in Figure 7. The calibration curve obtained with hippuric acid can be consulted on Appendix B (Page 27).

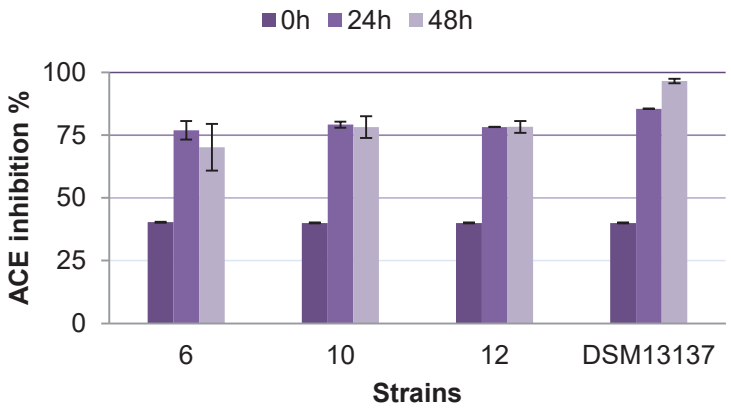

Figure 7. Results of ACE inhibitory activity in fermentation samples of three strains (6, 10, 12) of Chiapas cheese and DSM13137.

At 0h fermentation, fermented milk interestingly showed 40.3% inhibition throughout the isolates. This could be due to the production of ACE-inhibitory peptides during the pre-culture medium or/and because the samples were only taken after the inoculation of all the strains in milk, which took at least 15 minutes. It has also been reported that unfermented milk apparently exhibited slight ACE inhibitory activity and the ACE inhibitory activity was markedly increased throughout fermentation by probiotics LAB [55].

During fermentation, the percentage of ACE inhibition in samples with the selected isolates increased continuously reaching a maximum at 24h fermentation, with values between 76-79%.

On the other hand, *Lactobacillus helveticus* DSM13137 showed an increasing %ACE inhibitory activity after 24h. At 48h of fermentation, it was observed the highest ACE inhibitory activity (96.5%) in this study. These results are in accordance with earlier reports performed by Gonzalez (2011) [44].

These results clearly indicate that fermentation plays an important role in the release of ACE inhibitory compounds by these microorganisms.

Although the samples of the isolates did not show an inhibition higher than the comparative strain, these naturally occurring ACE-inhibitory peptides are reported to be advantageous over

the artificially synthesized ACE-inhibitory drugs (vasodilators, diuretics, calcium channel blockers, angiotensin II receptor blockers and ACE-inhibitors such as captopril, enalapril, alecepril, lisinopril) [36].

4.2.2. Antioxidant activity

The antioxidative potential of LAB has been reported in several studies but a single mechanism or compound associated with it has not been able to distinguish [56, 57]. As various methods are used to test the antioxidant activity the results are expressed in a variety of ways which makes comparison difficult.

In this study, the antioxidant properties of the fermented samples were evaluated by two different methods: ferric reducing antioxidant power (FRAP) and trolox equivalent antioxidant capacity (TEAC).

FRAP values were obtained by comparing the absorbance change at 595 nm in test reaction mixtures with those containing ferrous ions in known concentration. The values clearly increased with fermentation time due to the formation of the complex, as presented in Figure 8. A calibration curve obtained with ascorbic acid can be consulted on Appendix B (Page 27).

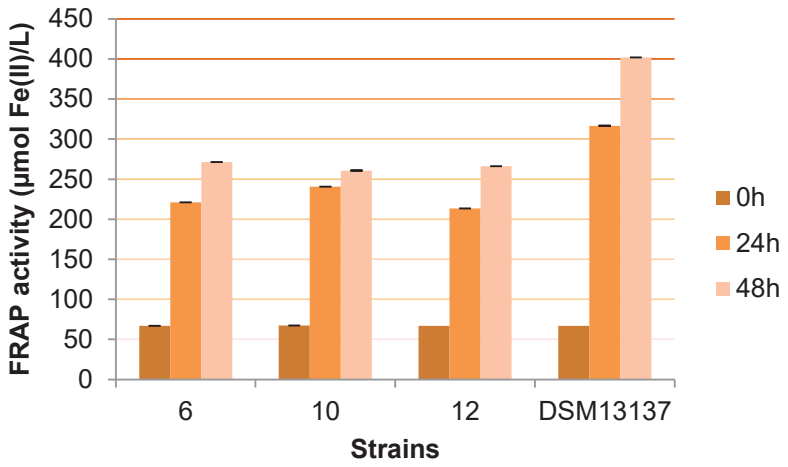

Figure 8. FRAP activity measured after 0, 24 and 48h of fermentation of three strains (6, 10, 12) of Chiapas cheese and DSM13137.

Higher FRAP values give higher antioxidant capacity because FRAP value is based on reducing ferric ion, where antioxidants are the reducing agent. Hence, these strains should be able to donate electrons to free radicals stable in the actual biological and food system. According to Hodzic *et al.* (2009), FRAP assay had been used to determine antioxidant activity as it is simple and quick [58]. Besides that, the reaction is reproducible and linearly related to molar concentration of the antioxidants.

Through the TEAC assay, the antioxidant activity was measured by the ability of whey to scavenge $ABTS^{\cdot+}$ cation radicals. In this method, the extent of scavenging of a preformed free radical relative to that of a standard antioxidant compound (Trolox, an analogue of Vitamin E) is determined.

The antioxidant activity of the different isolates varied between 292 and 1700µmol·L^{-1} (TEAC), as presented in Figure 9. A calibration curve with different known concentrations of Trolox can be consulted on Appendix B (Page 27).

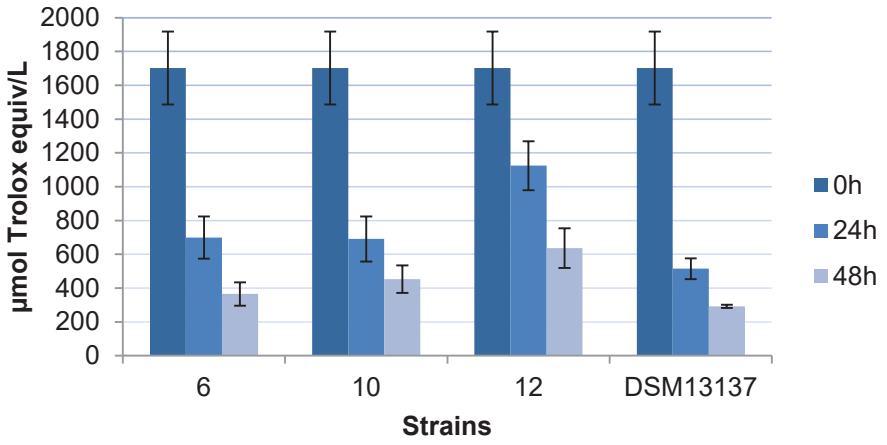

Figure 9. Results of TEAC assay in fermentation samples of three strains (6,10, 12) of Chiapas cheese and DSM13137.

All cell free samples showed highly scavenging potential against ABTS radical cation, ranging from 50 to 86%. The maximum activity was observed with DSM13137(comparative strain) after 48h of fermentation, followed by strain 6 (83.5%) as presented in Figure 10.

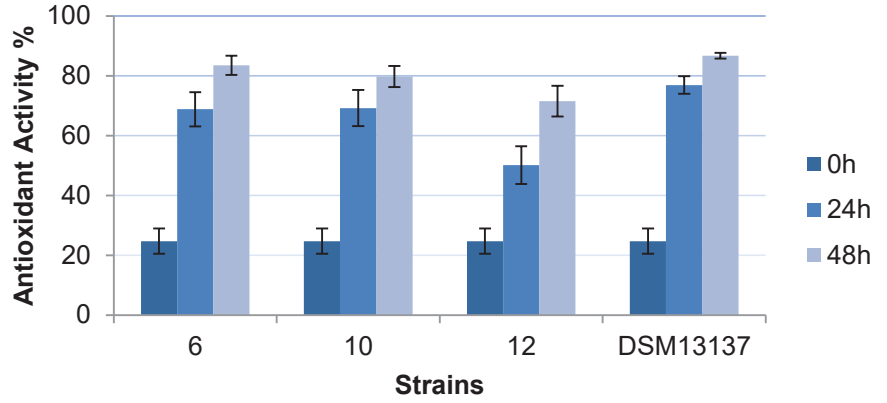

Figure 10. Results of antioxidant activity in fermentation samples of three strains (6, 10, 12) of chiapas cheese and DSM13137.

Both methods showed that antioxidant activity increased in all strains during fermentation. The milk fermented by *Lactobacillus helveticus* DSM 13137, which exhibited an intermediate proteolytic activity (DH 12%), the lowest between these three testing bacteria, showed the highest values of antioxidant activity in both methods. These results are in agreement with

those reported by Virtanen *et al.* (2006) which revealed the relationship between the development of antioxidant activity and a high degree of proteolysis [59].

Also, since the bacterial cells and most casein were removed from fermented samples these results confirm that the antioxidant activity obtained is originated from cell lysis products, extracellular metabolites or hydrolysed milk components.

4.2.3. Antimicrobial activity

The selected strains were examined according to their antimicrobial activity. For this purpose, cell-free neutral supernatant broths of these isolates were assessed against the different indicator microorganisms: *Escherichia coli* K12, *Salmonella enterica* serovar Typhi, *Salmonella enterica* serovar Enteritidis, *Listeria monocytogenes* 10403S, *Listeria monocytogenes* EGD-e and *Listeria monocytogenes* L028.

The diameter of inhibition zones (Table 3) showed that not all the isolates have antibacterial effect on the target microorganisms. Positive results are highlighted in grey.

Table 3. Results of the antimicrobial activity of 7 unknown strains isolated from Chiapas cheese and *Lactobacillus helveticus* DSM 13137.

	10403S	EGD-e	L028	K12	TYPHI	ENTERITIDIS
6	-	-	-	12.0±0.1	10.2±0.1	12.1±0.1
10	-	-	-	-	-	-
12	-	-	-	-	-	-
28	-	-	-	-	-	-
43	-	10.0±0.1	-	-	8.0±0.1	12.0±0.1
46	-	8.2±0.1	-	-	10.1±0.1	10.0±0.1
48	-	-	-	-	-	-
DSM13137	-	-	-	-	-	-

After 18h, strain 6 showed resistance to *Escherichia coli* K12, *Salmonella enterica* serovar Enteritidis and *Salmonella* Typhi, and strain 43 and 46 showed resistance to *Listeria monocytogenes* EGD-e, *Salmonella* Typhi and *Salmonella* Enteritidis. These results suggest that at least three strains isolated from Chiapas cheese have the ability to produce bacteriocins or other bioactive peptides with antimicrobial activities.

Although disc diffusion is a good method to screen the ability of potential metabolites to inhibit bacterial growth, it has some drawbacks. The interpretation of the results is mainly visual not having in consideration the activity undetected by the naked eye and also is based on the assumption that all metabolites diffuse freely in the solid nutrient medium which may lead to inaccurate assessment of bacterial susceptibility.

4.3 Molecular analysis

Although identification of the isolates was not performed in due time, a random amplified polymorphic DNA (RAPD) PCR was performed in order to identify genetic variation. This procedure detects nucleotide sequence polymorphisms in a DNA amplification-based assay using only a single primer of arbitrary nucleotide sequence [60]. In this reaction, a single species of primer binds to the genomic DNA at two different sites on opposite strands of the DNA template. If these priming sites are within an amplifiable distance of each other, a discrete DNA product is produced through thermocylic amplification. The polymorphisms between individuals result from sequence differences in one or both of the primer binding sites, and are visible as the presence or absence of a particular RAPD band [60].

In this study, the selected primer was OPA-09 and a dendogram with the RAPD-PCR patterns of each isolate is presented in Figure 11. The 84 isolates were organized in clusters according to the similarity of band profiles.

RAPD analysis only allows a qualitative assessment of the DNA effects and the nature of the changes in profiles can only be speculated unless amplicons are analysed (with sequencing, probing, etc.). Highlighted in blue are groups of isolates that could possibly be replicas of the same strain due to the similar band pattern obtained.

For a long time bifidobacteria have been considered relatives to lactobacilli, and the correlation was supported by the analysis of the murein structure [62]. In addition they shared other characteristics such as being Gram-positive, nonspore-forming, anaerobic, catalase negative and the ability to produce acid. However, Poupard *et al*. (1973) and other researchers were the first to postulate that they are more related to *Actinomyces*, which has been confirmed by analysis of 16S rRNA sequences [63, 64].

A Bifidobacterium genus specific PCR was performed but no bands with the expected size were found, hence no isolates belonged to this group as expected. The primer set used have a correspondence close to 100% in some studies being highly specific for its group [65]. The initial methods used for LAB isolation were effective.

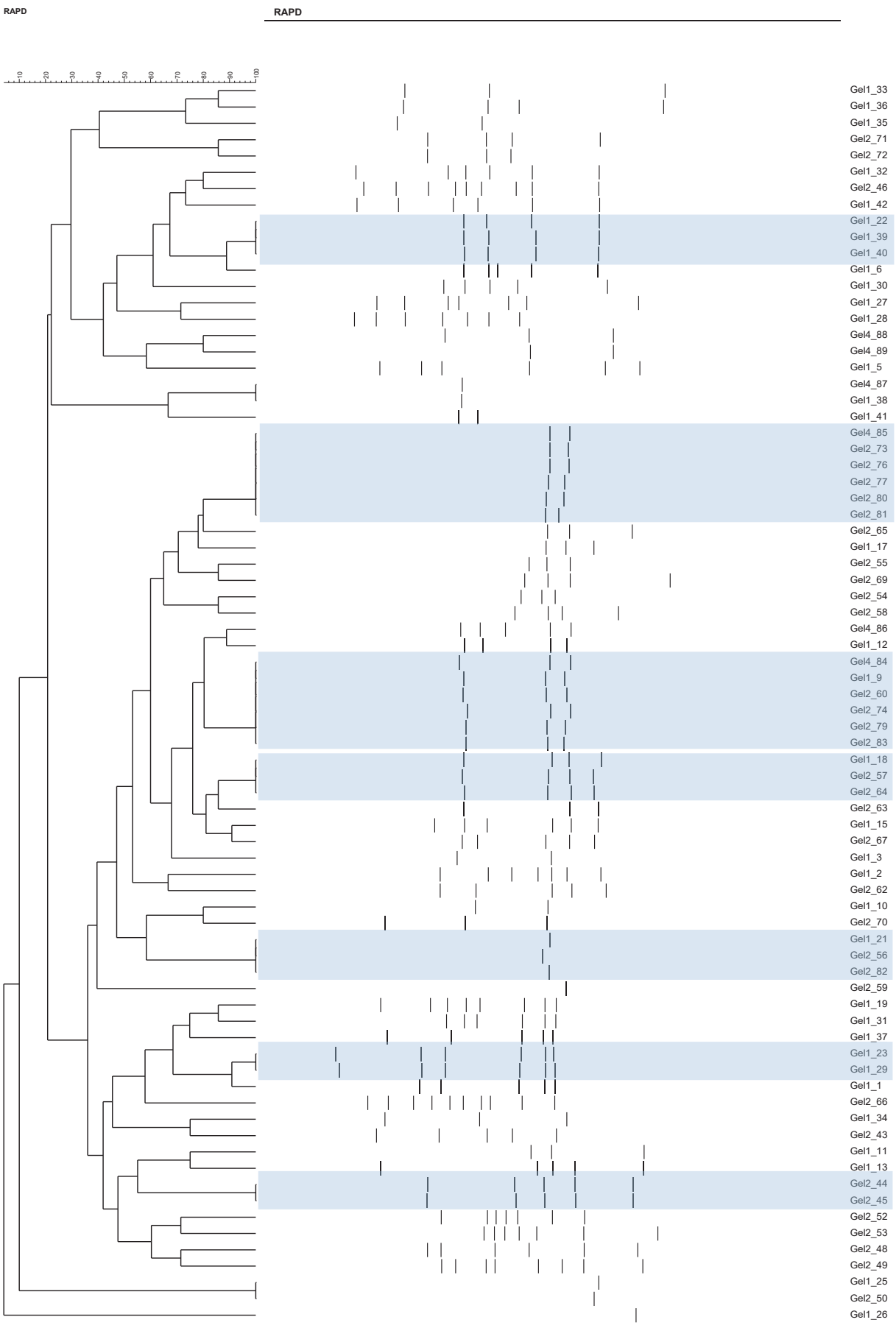

Figure 11. Dendogram of RAPD-PCR patterns with primer OPA-09 of 84 unknown isolates from Chiapas cheese generated by Gel Compar II.

5. Conclusion

With the characterization methods chosen it was possible to limit our focus on 24 isolates (from the 84). The determination of the degree of hydrolysis showed 7 strains with a better activity than *Lactobacillus helveticus* DSM13137. From the three isolates studied, strain 6 was the most promising. Not only revealed high antioxidant activity and the ability to produce ACE inhibitors during milk fermentation but also showed activity against pathogenic bacteria. The applications of these bacteria could be as starters in dairy industry due to their high hydrolytic profile and as preventing human diseases in which free radicals are involved, such as cancer, cardiovascular disease, and aging or even for cheese preservation. The search for natural ACE inhibitors as alternatives to synthetic drugs is of great interest to prevent several side effects, and bioactive peptides are one among the best alternatives which can be developed as potential pharmaceutical ACE inhibitors.

Although further studies and identification of the strains would be beneficial in the future, it is clear that traditional fermented products constitute a source of new strains of LAB that could be applied in functional and/or pharmaceuticals due to their properties.

Furthermore, the dairy industry which uses *Lactobacillus* species as starter cultures in dairy products, should work closely with the medical professionals in order to substantiate the health claims associated with these beneficial microorganisms.

6. Future directions

Lactic acid bacteria seem to be promising candidates in the preparation of nutraceuticals. Therefore, identification and/or modification of endogenous lactic acid bacteria from artisanal products with improved properties for this purpose is an obvious future objective.

Additional studies to these isolates should be performed in order to: identify the predominant peptides in the hydrolysates; stability to digestion - with a view to determine if the hydrolysates when subjected to simulating gastrointestinal conditions still show high activity. Also, antihypertensive peptides are only minor constituents in highly complex food matrices and, therefore, a monitoring of the large-scale production by hydrolytic or fermentative industrial process is also mandatory.

7. Appendix

Appendix A

Table 4. Summary of the isolates pre-screening assays.

Sample Code	Chiapas cheese sample	Atmosphere growth	Catalase	Diacetyl production	Total amount of Protein (mg/mL)	% Degree of Hydrolysis
1	Tabasco	Aerobic	-	-	*	*
2	Tabasco	Aerobic	-	+	3.737±0.004	11.26±0.06
3	Tabasco	Aerobic	-	-	*	*
4	Tabasco	Aerobic	-	-	*	*
5	Tabasco	Aerobic	+	+	*	*
6	Tabasco	Aerobic	-	+	12.500±0.003	23.64±0.12
7	Veracruz	Aerobic	+	-	*	*
8	Veracruz	Aerobic	+	-	*	*
9	Veracruz	Aerobic	-	-	*	*
10	Veracruz	Aerobic	-	+	6.300±0.001	18.02±0.01
11	Veracruz	Aerobic	+	-	*	*
12	Veracruz	Aerobic	-	+	6.638±0.006	14.99±0.02
13	Veracruz	Aerobic	+	-	*	*
15	Veracruz	Aerobic	-	-	*	*
16	Veracruz	Aerobic	-	-	*	*
17	Veracruz	Aerobic	-	-	*	*
18	Veracruz	Aerobic	-	-	*	*
19	Tabasco	Aerobic	-	-	*	*
21	Tabasco	Aerobic	+	-	*	*
22	Tabasco	Aerobic	-	-	*	*
23	Tabasco	Aerobic	-	-	*	*
24	Tabasco	Aerobic	-	-	*	*
25	Tabasco	Aerobic	-	-	*	*
26	Tabasco	Aerobic	-	-	*	*
27	Tabasco	Aerobic	-	+	3.30±0.005	12.20±0.01
28	Tabasco	Aerobic	-	+	4.688±0.004	15.32±0.04
29	Tabasco	Anaerobic	-	-	*	*
30	Tabasco	Anaerobic	-	-	*	*
31	Tabasco	Anaerobic	-	+	6.650±0.009	1.17±0.01
32	Tabasco	Anaerobic	-	+	7.313±0.004	3.42±0.03
33	Tabasco	Anaerobic	-	+	5.600±0.008	2.62±0.03
34	Tabasco	Anaerobic	-	-	*	*
35	Tabasco	Anaerobic	-	+	5.088±0.009	6.20±0.05
36	Tabasco	Anaerobic	-	+	4.675±0.006	5.06±0.02
37	Tabasco	Anaerobic	-	-	*	*
38	Tabasco	Anaerobic	-	-	*	*
39	Tabasco	Anaerobic	-	+	3.280±0.003	7.99±0.06
40	Tabasco	Anaerobic	-	+	3.063±0.001	6.19±0.05
41	Tabasco	Anaerobic	-	-	*	*
42	Tabasco	Anaerobic	-	-	*	*
43	Tabasco	Anaerobic	-	+	3.263±0.006	14.03±0.05
44	Tabasco	Anaerobic	-	+	3.688±0.009	11.63±0.05
45	Tabasco	Anaerobic	-	+	5.150±0.005	10.58±0.04
46	Tabasco	Anaerobic	-	+	5.538±0.006	14.51±0.05
47	Tabasco	Anaerobic	-	-	*	*
48	Tabasco	Anaerobic	-	+	4.375±0.003	14.28±0.05
49	Tabasco	Anaerobic	-	-	*	*
50	Tabasco	Anaerobic	-	+	3.813±0.008	7.30±0.02
51	Tabasco	Anaerobic	-	+	4.525±0.005	5.72±0.03
52	Tabasco	Anaerobic	-	-	*	*

53	Tabasco	Anaerobic	-	-	*	*
54	Tabasco	Anaerobic	-	-	*	*
55	Veracruz	Anaerobic	-	-	*	*
56	Veracruz	Anaerobic	-	-	*	*
57	Veracruz	Anaerobic	-	-	*	*
58	Veracruz	Anaerobic	-	-	*	*
59	Veracruz	Anaerobic	-	-	*	*
60	Veracruz	Anaerobic	-	-	*	*
61	Veracruz	Anaerobic	-	-	*	*
62	Veracruz	Anaerobic	-	-	*	*
63	Veracruz	Anaerobic	-	-	*	*
64	Veracruz	Anaerobic	-	-	*	*
65	Veracruz	Anaerobic	-	-	*	*
66	Veracruz	Anaerobic	-	-	*	*
67	Veracruz	Anaerobic	-	-	*	*
68	Veracruz	Anaerobic	-	-	*	*
69	Veracruz	Anaerobic	-	-	*	*
70	Veracruz	Anaerobic	-	-	*	*
71	Veracruz	Anaerobic	-	+	3.35±0.003	8.29±0.02
72	Veracruz	Anaerobic	-	-	*	*
73	Veracruz	Anaerobic	-	-	*	*
74	Veracruz	Anaerobic	-	-	*	*
75	Veracruz	Anaerobic	-	+	3.421±0.008	5.30±0.05
76	Veracruz	Anaerobic	-	+	4.863±0.008	0.33±0.05
77	Veracruz	Anaerobic	-	-	*	*
79	Veracruz	Anaerobic	-	-	*	*
80	Veracruz	Anaerobic	-	-	*	*
81	Veracruz	Anaerobic	-	-	*	*
82	Veracruz	Anaerobic	-	-	*	*
83	Veracruz	Anaerobic	-	-	*	*
84	Veracruz	Anaerobic	-	-	*	*
85	Veracruz	Anaerobic	-	-	*	*
86	Veracruz	Anaerobic	-	-	*	*
87	Veracruz	Anaerobic	-	-	*	*

Samples 14, 20 and 78 showed no growth therefore they were not used in this project.

- = negative result; + = positive result; * = Isolate not selected for further studies.

Appendix B

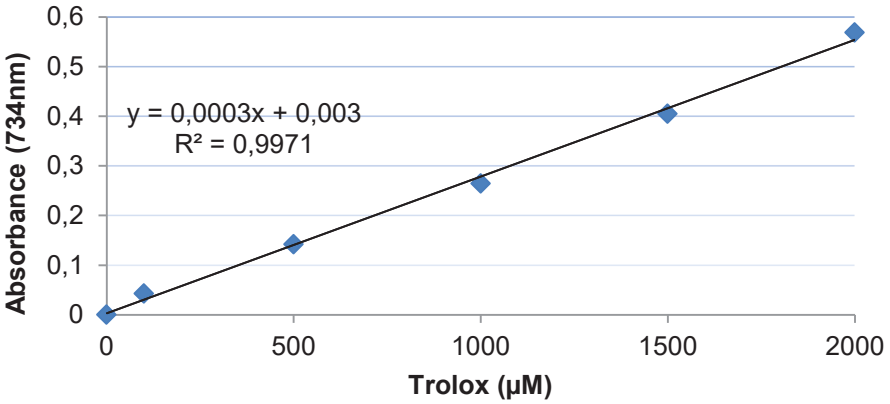

Figure 12. Calibration curve of Trolox in TEAC assay.

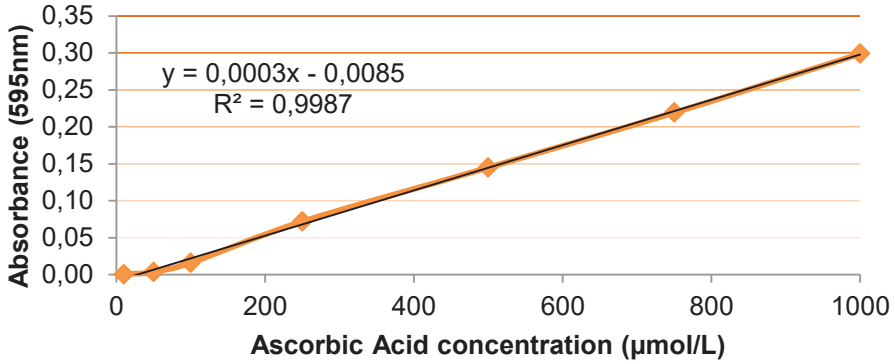

Figure 13. Calibration curve of Ascorbic acid in FRAP assay.

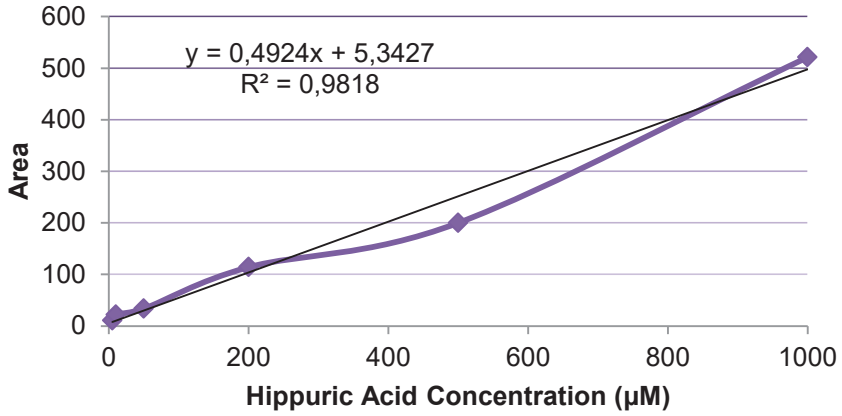

Figure 14. Calibration curve of Hippuric acid in ACE-inhibitory assay.

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