Highly proteolytic bacteria from semi-ripened Chiapas cheese elicit angiotensin-I converting enzyme inhibition and antioxidant activity

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Article

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Highlights

- Mexican Chiapas cheese shows high proteolytic activity by native bacteria.
- Chiapas cheese showed up to 0.78 g/kg of antihypertensive GABA content.
- Chiapas cheese elicit angiotensin-I converting enzyme inhibition.
- Fermented milk of selected isolates induced ACE inhibitory & antioxidant activities.

Highly proteolytic bacteria from semi-ripened Chiapas cheese elicit angiotensin-I converting enzyme inhibition and antioxidant activity

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Abstract[1]

Chiapas cream cheese (CCH) manufacturing process involves a long acid-enzymatic coagulation period of full-fat cow raw milk to achieve an acid and crumbly cheese. These sensorial aspects are related to lactic acid bacteria activity during ripening. Our main objective was to test the hypothesis that CCH contained highly proteolytic strains able to release bioactive compounds upon milk-protein hydrolysis. First, the proteolysis of CCH was evaluated considering the peptide and amino acid profiles of cheese samples collected from Veracruz (AVCH) and Tabasco (HTCH). The angiotensin-converting-enzyme (ACE) inhibitory activity in cheese water-soluble fractions was evaluated. Thereafter, strains from both CCH samples were isolated and selected based on their proteolytic capability, genetic fingerprint differentiation and growth conditions. Finally, a range of activities *in vitro* were tested in milk fractions

[1] ABBREVIATIONS:
2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{+\bullet}$); Acayucan Veracruz Chiapas Cheese (AVCH); angiotensin-I converting enzyme (ACE); ACE-inhibitory activity (ACEi); ACE-inhibitory efficiency ratio (IER); antioxidant activity (AO); antioxidant activity/total protein efficiency ratio (AOER); Bifidobacterium (B.); brain heart infusion (BHI); Chiapas cream cheese (CCH); Degree of hydrolysis (DH%); free amino acids (FAA); N-[3-(2-furyl)acryloyl]-Phe-Gly-Gly (FAPGG); fermented milk soluble fractions (FMSF); ferric reducing antioxidant power (FRAP); γ-aminobutyric acid (GABA); Huimanguillo Tabasco Chiapas Cheese (HTCH); Lactobacillus (Lb.); lactic acid bacteria (LAB); phosphate buffered saline (PBS); Man Rogosa Sharpe (MRS); o-phthaldialdehyde (OPA); reconstituted skim milk (RSM); reversed-phase high performance liquid chromatography (RP-HPLC); trolox equivalent (TE); trolox equivalent antioxidant capacity (TEAC); water soluble fractions (WSF).

fermented with selected strains. CCH showed ACE inhibitory activity: IC_{50}=1.75-2.75 mg/mL. Interestingly, AVCH contains 0.78 g/kg of the antihypertensive γ-aminobutyric acid. Three highly proteolytic strains showed ACE and high antioxidant activities upon milk fermentation. In conclusion, CCH contain proteolytic strains able to release bioactive compounds from milk proteins and potentially useful to produce functional ingredients and foods.

Keywords:

Chiapas cheese; proteolysis; GABA; ACE-inhibitory activity; antioxidant

1. Introduction

Chiapas cream cheese (CCH) is a semi-ripened traditional cheese manufactured in the tropical south of Mexico. It is characterised by its acidic taste and creamy sensory properties (i.e. mouth feel). These characteristics are conferred during the process that involves 3-5 h of whole raw milk maturation followed by 2- 8 h of coagulation and acidification by endogenous lactic acid bacteria (LAB) at tropical conditions (>25°C) (González-Córdova et al., 2016). CCH was reported a humidity of 48%, a pH about 4.0, and 5% of NaCl (Morales, Morales, Hernández, & Hernández-Sánchez, 2011). LAB are able to release bioactive compounds from milk proteins by proteolysis during cheese ripening. For instance, *Lactobacillus (Lb.) helveticus* DSM13137, a proteolytic cheese starter, releases the antihypertensive peptides Ile-Pro-Pro and Val-Pro-Pro during milk fermentation (Seppo, Jauhiainen, Poussa, & Korpela, 2003). The ACE inhibitory activity (ACEi) has been attributed as one of the main antihypertensive mechanisms of bioactive peptides. ACEi was reported in ripened Red Cheddar and Camembert with an IC_{50}, the amount of protein to inhibit the ACE activity by 50%, as low as 0.16 mg/mL, whereas non activity was detected in cottage, an unripen cheese (Okamoto et al., 1995). Furthermore, Gupta, Mann, Kumar & Sangwan (2013) showed a clear relationship between degree of hydrolysis (DH) and an increase of ACEi in cheese water-soluble fraction (WSF) when adding adjunct cultures at different stages of ripening Cheddar cheeses. Also, a positive correlation between ripening and the radical scavenging capability of Cheddar WSF was found when adding *Lb. casei* ssp *casei* 300 as adjunct culture (Gupta, Mann, Kumar, & Sangwan, 2009). Indeed, it was reported that CCH possess higher

antioxidant activity (AO) than other Mexican cheeses when assessed by the oxygen radical absorbance capacity (ORAC) method, suggesting that greater proteolytic activity in CCH compared to other cheese released more antioxidant compounds (Santiago-López et al., 2018). LAB strains with potential proteolytic activity have been reported in CCH (Morales et al., 2011). Also, CCH halotolerant strains *Lb. plantarum, Lb. pentosus, and Lb. acidipiscis* have shown probiotic characteristics such as antimicrobial activity and adhesion to mucin (Melgar-Lalanne, Rivera-Espinoza, Reyes Méndez, & Hernández-Sánchez, 2013). Nonetheless, no studies have been published on the proteolytic activity of the microbiota contained in CCH and their ability to releasing bioactive compounds. Thus, the main objective of this study was to investigate a range of potential functionalities, i.e. antihypertensive, and /or antioxidant activities, associated to the proteolytic activity of the microbiota present in CCH. Our first approach was to study the proteolysis occurred in CCH from two different regions considering the peptide and amino acid profile. Also, ACEi of cheese WSF was assessed. Subsequently, isolated strains from CCH samples were selected based on their proteolytic activity, ability to produce diacetyl and no catalase production and genetical fingerprint differentiation by using RAPD-PCR technique. ACEi and antioxidant activities were investigated in whey fermented with selected strains, which could potentially be used in the formulation and production of functional foods.

2. Material and methods

2.1 Reagents and cheese sample preparation and characterisation

Unless otherwise stated chemicals and reagents were obtained from Sigma-Aldrich, UK. Cheese samples labelled as "Queso Chiapas doble crema" were purchased from Mexican local markets at Acayucan Veracruz (AVCH) and Huimanguillo Tabasco (HTCH) from recent manufacture (1 week, according to the date of production labelled on the package) and stored at 5 °C. Samples for microbial isolation and bioactivity assessment were diluted in phosphate buffered saline (PBS); (0.01 M phosphate buffered saline (NaCl 0.138 M; KCl - 0.0027 M); pH 7.4, at 25 °C, 10% w/v). For amino acid analysis, samples were diluted in 0.1 N HCl (10%, w/v) and vortexed until complete dissolution. Diluted samples to be assessed for bioactivity were stored at -20 °C. For pH determination 1 g of cheese was

homogenised by using a vortex in 10 mL of distilled water and measure in a pH meter Hannah Instruments pH 211.

2.2 Determination of total protein, degree of hydrolysis and peptide profile

The total amount of proteins (TP) was determined using bicinchoninic acid as described by Gonzalez-Gonzalez, Tuohy & Jauregi (2011). The degree of hydrolysis (DH%) was determined using the method of o-phthaldialdehyde (OPA) described by Nielsen, Petersen & Dambmann (2001) and modified by Gonzalez-Gonzalez et al. (2011). The peptide profile was determined by reversed-phase high performance liquid chromatography (RP-HPLC) using a gradient as described by Gonzalez-Gonzalez, Gibson & Jauregi (2013).

2.3 Amino acid profile of cheese samples

The free amino acid (FAA) profiles of cheese samples, including γ-aminobutyric acid (GABA), were examined using a derivatisation assay kit EZ-Faast (Phenomenex USA), and running on a GC- Agilent 6890 GC-5975-MS system (Agilent, USA) in electron impact mode. The method used is based on Elmore, Koutsidis, Dodson, Mottram, & Wedzicha (2005). The results were compared to those found in raw milk.

2.4 Isolation of bacteria

Bacterial isolation was carried out under both aerobic and anaerobic conditions at 37 °C by spread-plating ten-fold serial dilutions (prepared in half-strength peptone water) on appropriate agar plates; de Man Rogosa Sharpe (MRS) agar for aerobic cultivation and both MRS agar and Columbia blood agar for anaerobic cultivation (Oxoid Ltd., UK). One of each different colony morphotype from each plate was subcultured, together with randomly selected colonies to obtain an equal number of isolates from each plate (cheese, agar type and cultivation conditions). Colonies were subcultured on the same agar type and incubated for 48 h appropriately (aerobic/anaerobic) to obtain pure cultures. Purified isolates were then stored on cryogenic Microbank^{TM} beads (ProLab Diagnostics, UK) at -80 ^{o}C.

2.5 Genetic fingerprinting of bacterial isolates

DNA was extracted from each isolate using the phenol/chloroform method and randomly amplified polymorphic DNA-PCR (RAPD-PCR) employed to estimate the genetic variations of the isolates. The reaction mixture (25 µL) comprised 5 µL of 5x GoTaq Flexi buffer (Promega, UK), 2.5 µL of dNTPs (0.4 mmol/L of each of dATP, dCTP, dGTP, dTTP; Promega), 1.5 µL of $MgCl_2$ (25 mmol/L; Promega), 1 µL of primer OPA-09 (5'-GGGTAACGCC-3'; 20 pmol/mL; Sigma Genosys, UK), 1 µL of GoTaq DNA polymerase (1.5 U/ µL; Promega), 1 µL of template DNA (5 ng/µL) and 13 µL of sterile water. PCR was performed using Prime thermal cycler (with heated lid, 100 ºC; Techne) programmed for 40 cycles of denaturation (30 sec at 94 ºC), annealing (60 sec at 38 ºC) and extension (2 min at 72 ºC), with a final 10 min extension step (72 ºC). The reaction products were separated via agarose gel electrophoresis (1.5% in 1x TAE buffer [Fisher, UK] containing ethidium bromide [0.5 ng/mL]), using 1 Kb DNA ladder (Promega) as a molecular size indicator. DNA fragment patterns were visualized under UV light (Genesnap, Syngene) and analysed by Gel Compar II software.

2.6 Fermentation of milk by isolated strains, diacetyl and catalase test

Bacterial strains were reactivated on appropriate agar plates (aerobically/anaerobically), checked for purity (single colony type) and overnight brain heart infusion (BHI) broth cultures prepared (aerobically or anaerobically, as per original isolation). Milk fermentations were carried out in Hungate tubes with 15 mL of 10% (w/v) reconstituted skim milk (RSM) using 1% inoculum of fresh overnight broth culture for each isolate (affording initial number of 10^6-10^7 cells/mL). A negative control, using uninoculated BHI (1%) was also included. The fermentations were incubated at 30 ºC with continuous agitation for 24 h. Samples of 2 mL were then taken and heated to 72 ºC for 1 min to stop the enzymatic proteolysis and then centrifuged at 12,000 *g* for 10 min. The supernatant was filtered and stored at –20 ºC until further analysis.

The catalase test was performed by mixing a colony into a drop of 3% hydrogen peroxide. Diacetyl production was determined to the supernatant fermented whey fraction according to King (1948). A purple ring at the top of the solution indicated the presence of diacetyl.

2.7 Activity *in vitro* assays

Assays for ACEi was performed in cheese samples. Furthermore, the ACEi and antioxidant activities were performed on fermented milk soluble fractions (FMSF) of selected strains according to criteria discussed below in section 3.3.

ACE inhibitory activity

The ACEi of cheese samples was determined using N-[3-(2-furyl)acryloyl]-Phe-Gly-Gly (FAPGG) according to the method described by Henda et al. (2013) with some modifications. Briefly, in a microplate well 10 µL of ACE (250 mU solution with 0.05 M Tris, 0.3 M NaCl in 50% glycerol solution, with the pH adjusted to 7.5 with 5 M HCl) was mixed with 150 µL of 0.88 mmol/L FAPGG in 0.05 M Tris, 0.3 M NaCl and 10 µL of test sample (diluted cheese or fermented milk fraction). 0.05 M Tris, 0.3 M NaCl buffer was used as negative control and 5 M HCl was used as positive control (standard inhibition). The reaction kinetics was followed in a Tecan Microplate Reader – A-5082 Spectra FLUOR plus (Austria) for 30 minutes to obtain the slope inhibitor [FAPGG] vs time (min). The ACEi% was calculated in relation to the slope generated when no inhibitor was present in the reaction (slope blank) according to equation 1 (below).

Equation 1 $$ACEi\% = [1 - (\text{slope inhibitor}/\text{slope blank})] \times 100$$

The IC_{50}, defined as the concentration of protein needed to inhibit the activity of the enzyme by half, was calculated as the concentration needed to reduce the slope by 50% in relation to the slope blank. The ACEi% in fermented milk fractions with selected strains, as described above, was determined according to the HPLC method described by Gonzalez-Gonzalez et al. (2011) using Hip-His-Leu as substrate.

Antioxidant activity de FRAP

The ferric reducing antioxidant power (FRAP) assay, based on the reduction of Fe(III) to Fe(II) by the action of antioxidants present, was performed according to Benzie & Strain (1996). Serial dilutions of ascorbic acid were used as standards.

Antioxidant activity by ABTS+• radical

For the trolox equivalent antioxidant capacity (TEAC) assay a solution of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{+\bullet}$) radical was prepared according to the method described by Guo & Jauregi (2018). Triplicates of each reaction were read at 734 nm in a spectrophotometer Amersham Ultrospec 1100 Pro UV/Vis (Uppsala, Sweden). Trolox standards were used for quantification and data was expressed as µmol trolox equivalent (TE). The antioxidant activity (AA%) was calculated by using equation 2 (below).

Equation 2 $$AA\% = 1 - \frac{ABS_{sample}}{ABS_{control}} \times 100$$

Where $ABS_{control}$ is the absorbance of $ABTS^{+\bullet}$ in PBS and ABS_{sample} is the reaction absorbance with the sample.

2.8 Data analysis and statistics

All data analysis including one-way analysis of variance and Tukey post-hoc test were performed for comparative analysis among means for each bioactivity using R v.3.4.3. (R-Core-Team, 2017).

3. Results and Discussion

3.1 Proteolysis in Chiapas cheese

The proteolytic activity undergoing in CCH samples is depicted in the peptide profile chromatograms. Fig. 1 showed intact milk proteins in RSM as control (Fig. 1A), and a significant breakdown in AVCH (Fig. 1B) and HTCH (Fig. 1C) peptides profiles. Larger peak areas of peptides in AVCH suggests higher proteolytic activity than in HTCH. This is confirmed by the FAA profiles, which show higher concentrations of total FAA in AVCH (3.89 g/kg) than in HTCH (0.37 g/kg) (Table 2). These concentrations are similar to those found in Spanish cheese (0.19 and 69 g/kg) (Diana, Rafecas, Arco, & Quílez, 2014). Moreover, the essential amino acids (AA) leucine, phenylalanine, lysine and valine were found in important amounts as FAA in AVCH and much higher than in HTCH. Additionally, Ornithine

175 (Orn), a non-proteinogenic AA found in certain types of ripened and semi-ripened cheeses, was found in
176 AVCH samples (0.18 g/kg) but not in HTCH samples. These differences on the peptide and FAA
177 profiles may be explained by the activity of the microbiota of each sample as well as the differences in
178 the manufacturing process (Santiago-López et al., 2018). Also, other factors such as salt content, pH and
179 temperature storage may influence the release of FAA (Diana et al., 2014).
180
181 GABA is an important antihypertensive AA produced by LAB by the decarboxylation of glutamate. In
182 this study, GABA was 0.78 g/kg in AVCH (Table 2), being one of the highest GABA concentrations
183 reported in cheese made of bovine milk so far; it has been found in Gouda (0.177 g/kg), Cheddar (0.048
184 g/kg) and blue cheese (0.007 g/kg) (Nomura, Kimoto, Someya, Furukawa, & Suzuki, 1998). Diana et
185 al., (2014) reported GABA in Spanish cheeses in amounts ranging from 0.01 to 0.31 g/kg in cheese
186 made of cow's milk and 0.07 to 0.98 g/kg in cheese made of ewe's milk. Nejati et al. (2013) reported a
187 fermented milk using a selected *Lb. plantarum* PU11 yielding up to 0.14 g/kg GABA after 120 h of
188 fermentation. Also, Lacroix, St. Gelais, Champagne, & Vuillemard (2013) identified cheese starters
189 *Lactococcus lactis* with high GABA production yielding up to 3.4 g/kg of GABA in Danish Havarti
190 cheese with added culture, attributing this to an extensive ripening and proteolysis. Also, glutamic acid
191 was found in raw milk, yet it was not detected in cheese suggesting that it has been converted to GABA
192 by LAB. Moreover, a reduction in blood pressure in mild-hypertensive patients was achieved following
193 a 12-weeks intake of fermented milk with 0.010–0.012 g of GABA per day (Inoue et al., 2003). This
194 content of GABA would be equivalent to daily consumption of approximately 13 g of AVCH.
195

196 **3.2 ACE inhibitory activity *in vitro* in cheese samples**

197 ACE inhibitory peptides may be released during cheese ripening. Although AVCH showed lower ACE
198 inhibitory potency (i.e. higher IC_{50} value), no significant differences were found in ACEi between the
199 two cheese samples (Table 1). The ACEi has been investigated in the norwegian traditional cheese
200 Gamalost reporting IC_{50} values as low as 0.34 ± 0.07 mg/mL after 10 days of ripening with native
201 microbiota (Qureshi, Vegarud, Abrahamsen, & Skeie, 2012). In a cheddar cheese enriched with *Lb.*

202 *casei* subps *casei* IC_{50} values were as low as 0.160 ± 0.002 after 3 months of ripening (Ong, Henriksson,
203 & Shah, 2007). They observed that ACEi potency declines in cheese after a long period of ripening due
204 to further proteolysis of bioactives peptides into their constituent AAs. This may explain why AVCH
205 with higher proteolytic activity showed lower ACEi potency than HTCH samples.

206 **3.3 Isolation and selection of highly proteolytic strains**

207 Our research aim was to screen highly proteolytic native bacteria in CCH that were able to release
208 bioactive compounds. A total of 89 bacterial strains were isolated (anaerobically and aerobically) but
209 only 84 isolates were able to be subcultured and characterised. RAPD-PCR was performed to obtain a
210 simple genetic fingerprint for each isolate and thus identify genetic variation across the strains (Fig. 2).
211 The 84 isolates were also tested for catalase activity, diacetyl production and the final pH of fermented
212 RSM during pre-screening (Table 3). A single representative for each biotype (based on fingerprint and
213 similarities in the pre-screening characteristics: catalase negative isolates that were capable of producing
214 diacetyl were considered desirable) was used in subsequent analyses. A pH < 4.7 after 24 h of
215 fermentation is also desirable, as it may be indicative of lactic acid production. The pH of control RSM
216 was 6.50 ± 0.03. Eight isolates from CCH demonstrated higher proteolytic activity (DH% > 8.9%) than
217 *Lb. helveticus* DSM1313, used here as a reference commercial strain with high proteolytic activity
218 (Table 3). In addition, s6-HTCH with a pH > 6.0 but with the highest DH% (>17.88) was also selected.
219 The peptide profiles by RP-HPLC from s10-AVCH and s12-AVCH displayed more diverse and
220 abundant peaks (Fig. 3B and 3C), similar to the chromatogram of AVCH (Fig. 1B). The remaining six
221 strains which elicited higher DH% than DSM13137 were isolated from HTCH. Whilst, s6-HTCH
222 displayed the highest DH%, it did not show as many peaks as s10-AVCH or s12-AVCH. This could be
223 explained by the high final pH (kept above 6.4) for this strain which allowed the caseins to remain
224 soluble which resulted in more extensive hydrolysis as shown by the peptide profile between Rt 40 and
225 50 min (Fig. 3A) and the production of very small peptides or FAA that would have eluted with the
226 solvent and/or were at concentrations below the detection limits.

227 **3.4 Bioactivity assays in fermented milk by selected hydrolytic strains**

228 Three strains, s6-HTCH, s10-AVCH, s12-AVCH, with desirable characteristics isolated in aerobic
229 conditions were chosen for ACEi and antioxidant activity (AO). The inhibitory efficiency ratio (IER) is
230 the quotient of ACEi% divided by the TP concentration providing a better approach to the potency of
231 inhibition than just reporting ACEi%. The IER of DSM13137 increased over time and it was the
232 highest of all the fermented milk fractions (Table 4), followed by s10 and s12 which were not
233 significantly different ($P<0.01$) with IER ≈ 9. Interestingly, s6 showed the lowest activity among the 4
234 strains despite showing higher DH%. Moreover, IER value decreased after 48 h of fermentation
235 suggesting further breakdown of bioactive peptides by proteolysis into inactive AA.
236
237 LAB may also generate antioxidant peptides during fermentation (Virtanen, Pihlanto, Akkanen, &
238 Korhonen, 2007). In this study, the antioxidant activity of the fermented samples was evaluated by both
239 FRAP (Fig. 4A) and the ABTS methods (Fig. 4B). Both methods showed the highest AO for
240 DSM13137 closely followed by the three strains assessed. Also, there was an increase of AO
241 fermentation time for all strains at 48 hours compared to 24 hours. This positive correlation of AO with
242 degree of hydrolysis is supported by results previously reported where CCH showed higher antioxidant
243 activity with time of ripening (Aguilar-Toalá, Vallejo-Cordoba, Hernández-Mendoza, & González-
244 Córdova, 2015). The range of AO observed for ABTS scavenging, 50 to 87% (>1600 μmol/L TEAC),
245 is higher than that found on sweet whey (36%) and β-lactoglobulin (26%) hydrolysates obtained with
246 protease N 'Amano' after 6 hours of hydrolysis (Welderufael, 2011). Nevertheless, it is known that
247 caseins are more susceptible to proteolysis than whey proteins, resulting in an increased AO (Power,
248 Jakeman, & Fitzgerald, 2013). Moreover, Virtanen et al. (2007) reported AO as 860 μmol/L TEAC in
249 fermented milk by a combination LAB, including *Leuconostoc cremoris*, *Lactococcus lactis*
250 ATCC19435 and *Lb. acidophilus* ATCC4356. Soleymanzadeh et al. (2016) reported antioxidant activity
251 of *Leuconostoc lactis* SM10, isolated from a traditional fermented camel milk, obtaining 1484 and
252 311.66 μmol/L TEAC after 24 hours of fermentation of camel and bovine milk, respectively using the
253 ABTS method. Interestingly, the AO reported in this study for DSM13137 (2138.95 ±20.23 μmol/L
254 TEAC), followed by strain s6 (2059.06 ± 26.27 μmol/L TEAC) after 48 hours of fermentation are
255 higher than those previously reported. Overall, the three selected isolates from CCH (s6-HTCH, s10-

AVCH and s12-AVCH), as well as DSM13137, show great capability to elicit AO activity upon milk fermentation.

4. CONCLUSIONS

In this study we reported for the first time the proteolytic activity in Chiapas cheese (CCH), particularly from the Veracruz (AVCH) and Tabasco (HTCH) regions. There was high proteolytic activity in both cheeses, but as supported by both the free amino acids content and the peptide profile, higher proteolytic activity was found in AVCH than in HTCH. Interestingly AVCH contained high amounts of essential amino acids as FAA and the antihypertensive GABA (0.78 g/kg); this amount is greater than what has been found in other cheese made of cow's milk and indicates the presence of LAB with capabilities to synthesise GABA. Furthermore, the proteolytic capabilities of microbiota isolated from the CCH was also assessed and their proteolytic activity was found similar to the reference commercial strain DSM13137. Furthermore, two of the isolated strains showed similar ACE inhibitory activity to DSM13137, and all strains tested (s6-HTCH, s10-AVCH and s12-AVCH) showed very similar antioxidant activities to the reference strain and higher than those previously reported for fermented milk. Thus, these cheese isolates with high proteolytic activity could lead to the production of functional foods with a range of biological functionalities. Further research should aim at the identification of the selected proteolytic strains and identification of major peptides responsible for the bioactivities.

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Table1

Table 1. IC_{50} values for ACE inhibitory activity, protein content and pH for Chiapas cheese samples from Veracruz (AVCH) and Tabasco (HTCH) (n=2, ± s.e.).

Activity	**AVCH**	**HTCH**
ACE inhibition (IC_{50} mg/mL)	2.75 ± 0.50[a]	1.75 ± 0.49 [a]
Total protein (g/100g)	26.95 ± 3.44 [b]	23.72 ± 2.88 [b]
pH	3.90 ± 0.06 [c]	3.95 ± 0.09 [c]

[a,b,c] No statistical difference between the means with the same letter; α= 0.05.

Table2

Table 2. Levels of free derivatised amino acids in raw milk and Chiapas cheese samples from Veracruz (AVCH) and Tabasco (HTCH).

Amino acid	**Raw milk g/kg**	**AVCH g/kg**	**HTCH g/kg**
Alanine	0.005	0.283	0.072
α-aminobutyric acid	0.001	0.022	ND
Asparagine	Tr[1]	0.135	0.009
Aspartic acid	0.010	0.148	0.010
γ-aminobutyric acid	**ND[2]**	**0.784**	**0.160**
Glutamic acid	0.059	0.076	0.009
Glutamine	ND	0.038	ND
Glycine	0.008	0.095	0.020
Histidine	ND	0.003	ND
Isoleucine	0.001	ND	Tr
Leucine	0.001	0.757	0.085
Lysine	0.002	0.464	0.031
Methionine	Tr	0.157	ND
Ornithine	0.001	0.179	ND
Phenylalanine	0.001	0.223	0.032
Proline	0.003	0.070	0.064
Serine	Tr	0.147	ND
Threonine	0.001	0.089	ND
Tyrosine	0.001	0.016	0.019
Tryptophan	0.001	0.009	ND
Valine	0.004	0.204	0.028

[2]Tr, traces

[1]ND, None detected.

Table3

Table 3. Screening of lactic acid bacteria isolated from Chiapas cheeses from Veracruz (AVCH) and Tabasco (HTCH) for their catalase activity, diacetyl production and pH of fermented reconstituted skim milk (RSM) to determine those with desirable characteristics (DC).[†]

ID	Origin	Atmosphere	Catalase	Diacetyl	pH	DC	DH(%)
s1	HTCH	Aerobic	-	-	6.28		ND
s2	HTCH	Aerobic	-	+	5.29		8.03
s3	HTCH	Aerobic	-	-	5.50		ND
s4	HTCH	Aerobic	-	-	6.10		ND
s5	HTCH	Aerobic	+	+	5.71		11.11
s6	HTCH	Aerobic	-	+	6.46	*	17.88
s7	AVCH	Aerobic	+	-	6.65		ND
s8	AVCH	Aerobic	+	-	6.66		ND
s9	AVCH	Aerobic	-	-	6.58		ND
s10	AVCH	Aerobic	-	+	5.31	*	13.41
s11	AVCH	Aerobic	+	-	6.59		ND
s12	AVCH	Aerobic	-	+	5.54	*	11.00
s13	AVCH	Aerobic	+	-	6.70		ND
s15	AVCH	Aerobic	-	-	6.30		ND
s16	AVCH	Aerobic	-	-	6.74		ND
s17	AVCH	Aerobic	-	-	6.73		ND
s18	AVCH	Aerobic	-	-	6.65		ND
s19	HTCH	Aerobic	-	-	5.98		ND
s21	HTCH	Aerobic	+	-	6.77		ND
s22	HTCH	Aerobic	-	-	6.23		ND
s23	HTCH	Aerobic	-	-	6.09		ND
s24	HTCH	Aerobic	-	-	6.18		ND
s25	HTCH	Aerobic	-	-	6.10		ND
s26	HTCH	Aerobic	-	-	5.88		ND
s27	HTCH	Aerobic	+	+	5.49		8.78
s28	HTCH	Aerobic	-	+	4.63	*	11.26
s30	HTCH	Anaerobic			6.24		ND

s31	HTCH	Anaerobic	-	+	5.28		0.00
s32	HTCH	Anaerobic	-	+	6.62		1.79
s33	HTCH	Anaerobic	-	+	5.11		1.16
s34	HTCH	Anaerobic	-	-	5.71		ND
s35	HTCH	Anaerobic	-	+	4.80		4.01
s36	HTCH	Anaerobic	-	+	4.92		3.10
s37	HTCH	Anaerobic	-	-	6.11		ND
s38	HTCH	Anaerobic	-	-	6.08		ND
s39	HTCH	Anaerobic	-	+	5.98		5.43
s40	HTCH	Anaerobic	-	+	5.60		4.00
s41	HTCH	Anaerobic	-	-	5.94		ND
s42	HTCH	Anaerobic	-	-	6.44		ND
s43	HTCH	Anaerobic	-	+	4.70	*	10.24
s44	HTCH	Anaerobic	-	+	4.48		8.32
s46	HTCH	Anaerobic	-	+	4.83	*	10.62
s47	HTCH	Anaerobic	-	-	6.64		ND
s48	HTCH	Anaerobic	-	+	4.98	*	10.43
s49	HTCH	Anaerobic	-	-	4.06		ND
s50	HTCH	Anaerobic	-	+	5.38		4.88
s51	HTCH	Anaerobic	-	+	5.27		3.63
s52	HTCH	Anaerobic	-	-	4.21		ND
s53	HTCH	Anaerobic	-	-	4.50		ND
s54	HTCH	Anaerobic	-	-	6.60		ND
s55	AVCH	Anaerobic	-	-	5.92		ND
s58	AVCH	Anaerobic	-	-	6.10		ND
s59	AVCH	Anaerobic	-	-	6.80		ND
s60	AVCH	Anaerobic	-	-	6.60		ND
s61	AVCH	Anaerobic	-	-	6.80		ND
s62	AVCH	Anaerobic	-	-	6.81		ND
s63	AVCH	Anaerobic	-	-	6.73		ND
s65	AVCH	Anaerobic	-	-	6.81		ND

s66	AVCH	Anaerobic	-	-	6.26	ND
s67	AVCH	Anaerobic	-	-	6.93	ND
s68	AVCH	Anaerobic	-	-	6.60	ND
s69	AVCH	Anaerobic	-	-	6.13	ND
s70	AVCH	Anaerobic	-	-	5.91	ND
s71	AVCH	Anaerobic	-	+	6.16	5.67
s72	AVCH	Anaerobic	-	-	6.36	ND
s73	AVCH	Anaerobic	-	-	6.69	ND
s74	AVCH	Anaerobic	-	-	6.64	ND
s76	AVCH	Anaerobic	-	+	5.37	0.0
s86	AVCH	Anaerobic	-	-	6.14	ND

† (-) negative reaction; (+) positive reaction. pH of uninoculated RSM (control) was 6.50±0.03. Those strains showing an equivalent fingerprint have been omitted. DH(%), degree of hydrolysis of milk proteins (%); negative control (RSM) 3.18% and positive control (*Lactobacillus helveticus* DSM1317) 8.84%. The specific desirable characteristics were catalase negative, capable of diacetyl production and DH% > 8.9%.

Table4

Table 4. Inhibitory efficiency ratio (ACEi%/[total protein mg mL^{-1}]) of fermented milk whey samples of selected strains (s6-HTCH, s10-AVCH & s12-AVCH) compared to *Lb. helveticus* DSM13137. Results are represented by the mean ± SD (n=2).

Time (hours)	DSM13137	s6-HTCH	s10-AVCH	s12-AVCH
24	$12.32 \pm 0.01^{a,b}$	$4.61 \pm 0.49^{b,c}$	$9.75 \pm 0.31^{b,c}$	9.09 ± 0.01^{b}
48	$15.42 \pm 0.25^{a,b}$	$3.74 \pm 1.21^{b,c}$	$8.32 \pm 1.31^{b,c}$	$9.11 \pm 0.59^{b,c}$

* Treatments with the same letter are not significantly different, Tukey HSD test: α=0.05

Figure1

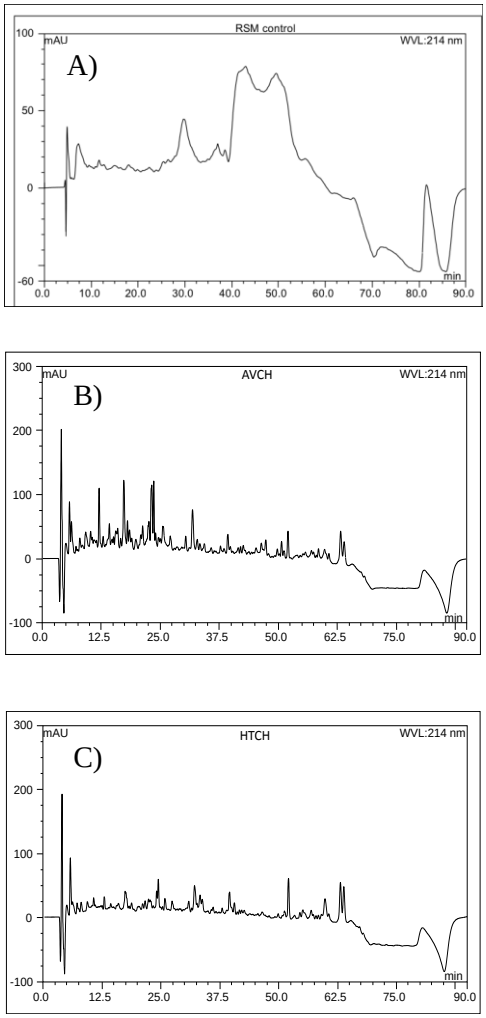

Fig. 1. RP-HPLC peptide profiles of A) reconstitute skimmed milk (RSM); B) Chiapas cheese from Veracruz (AVCH); and C) from Tabasco (HTCH).

Figure2

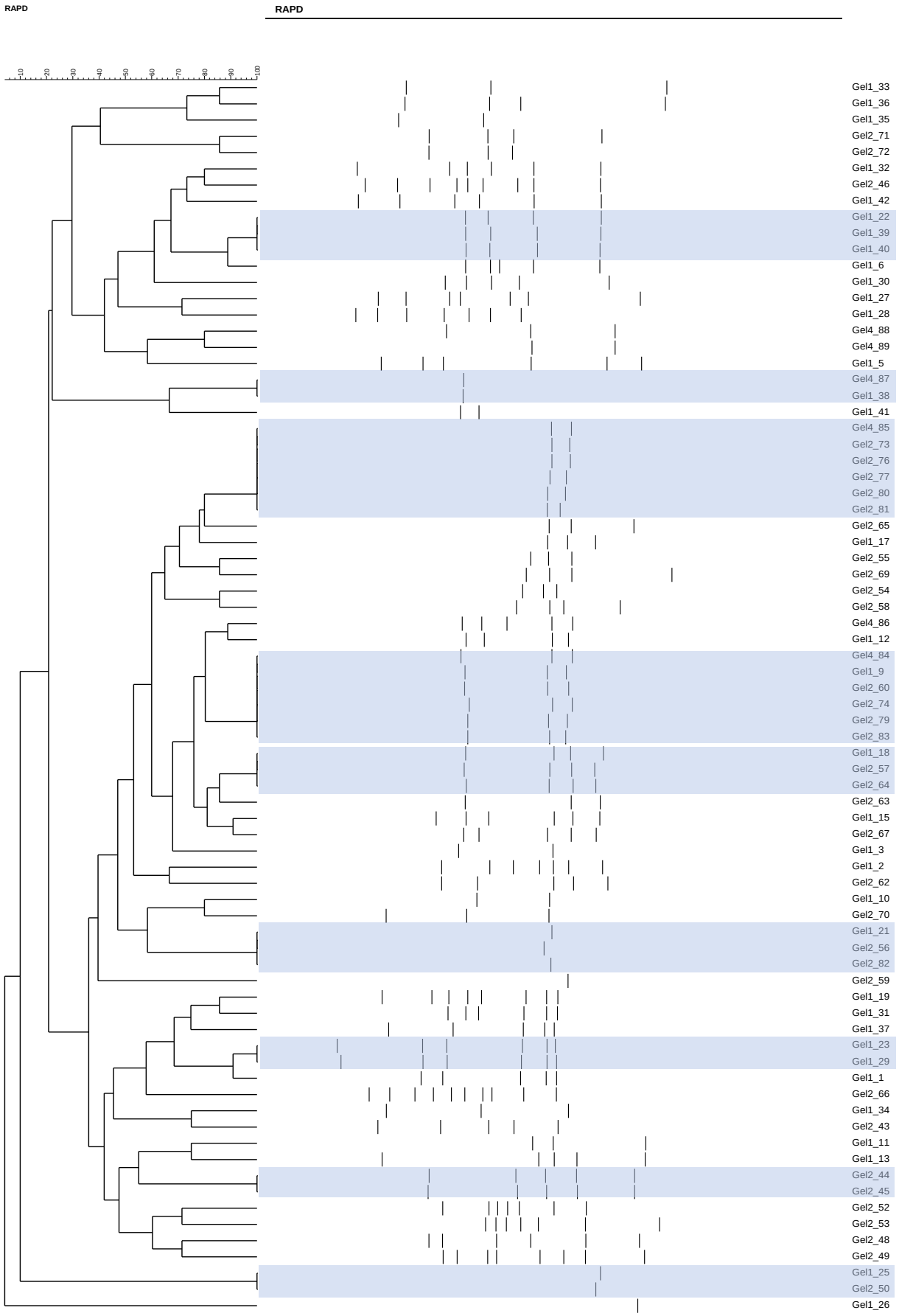

Fig. 2. Investigation of the genetic variation of lactic acid bacteria isolated from Chiapas cheeses using RAPD-PCR (OPA-09). Profiles are labelled with gel number followed by isolate number (e.g. Gel1_33, refers to isolate 33 whose RAPD-PCR product was run in Gel 1). Grey shading highlights potential replicates of the same strain (100% similarity between RAPD-PCR profiles).

Figure3

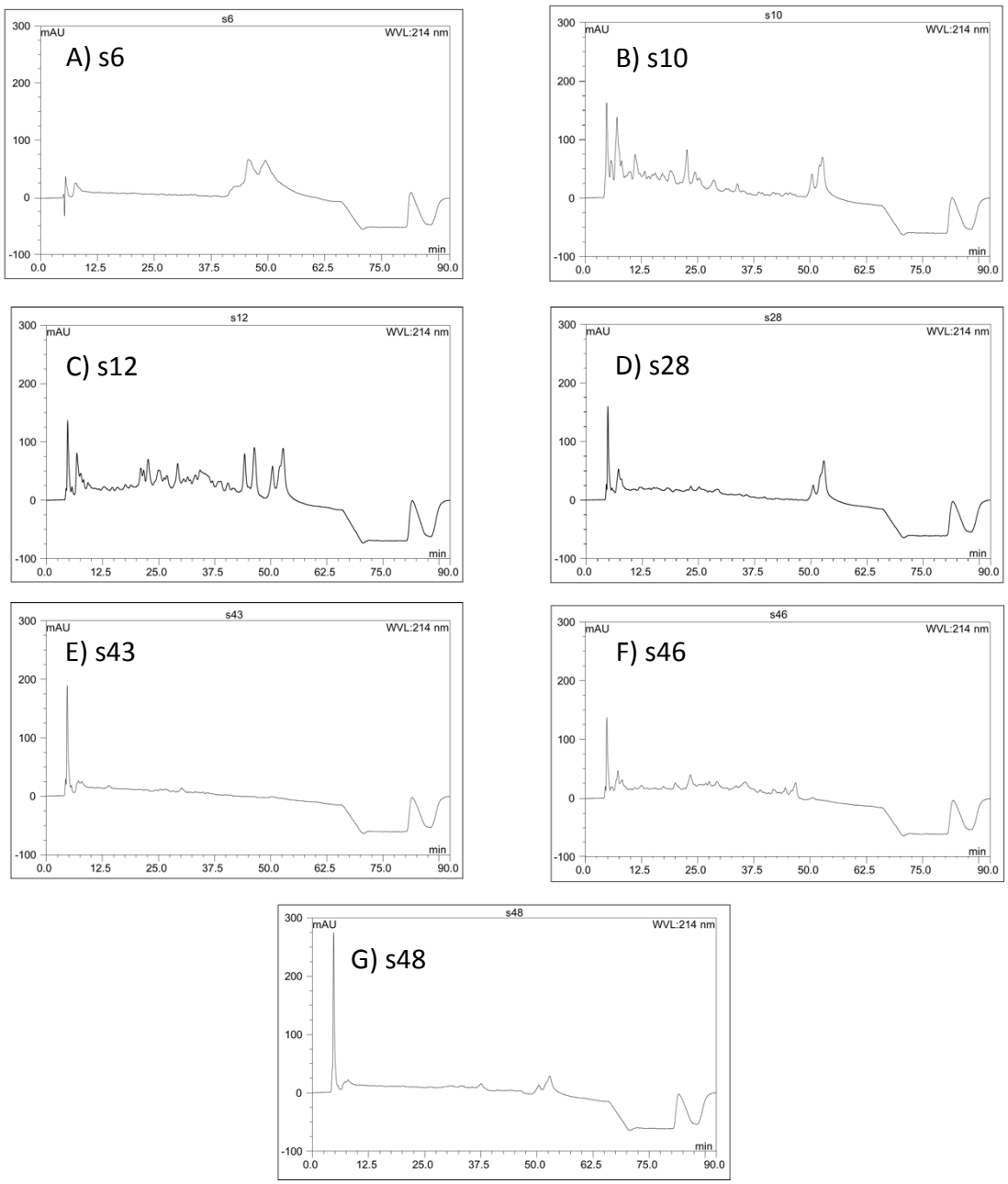

Fig. 3. Reverse phase chromatograms of peptide profiles of fermented milk whey fractions of lactic acid bacteria (s6-HTCH, s10-AVCH, s12-AVCH, s28-HTCH, s43-HTCH, s46-HTCH, and s48-HTCH) isolated from Chiapas cheese samples.

Figure4

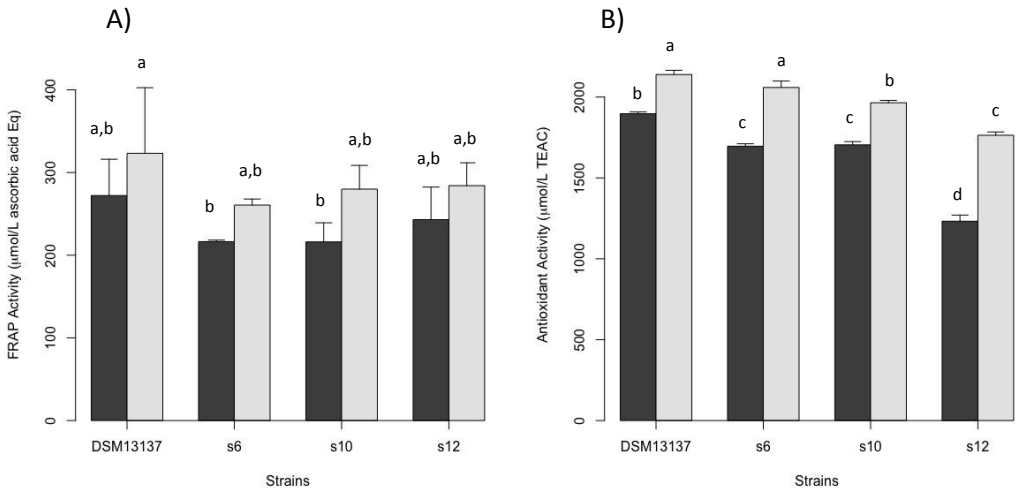

Fig. 4. Antioxidant activity of fermented milk whey fractions after 24 (dark grey) and 48 hours (light grey) fermentation by *Lactobacillus helveticus* DSM13137, s6-HTCH, s10-AVCH, and s12-AVCH. A) FRAP; and B) ABTS. Data are presented as means ± SD (n=3). Treatments with the same letter are not significantly different, Tukey HSD test: α=0.05