

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

Article

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Gonzalez-Gonzalez, C. R., Machado, J., Correia, S., McCartney, A. L., Elmore, J. S. ORCID: <https://orcid.org/0000-0002-2685-1773> and Jauregi, P. (2019) Highly proteolytic bacteria from semi-ripened Chiapas cheese elicit angiotensin-I converting enzyme inhibition and antioxidant activity. *LWT-Food Science and Technology*, 111. pp. 449-456. ISSN 0023-6438 doi: 10.1016/j.lwt.2019.05.039 Available at <https://centaur.reading.ac.uk/83763/>

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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.

# 1 **Highly proteolytic bacteria from semi-ripened Chiapas cheese elicit** 2 **angiotensin-I converting enzyme inhibition and antioxidant activity**

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## 9 **Abstract<sup>1</sup>**

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

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### <sup>1</sup> ABBREVIATIONS:

2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS<sup>+</sup>); 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);  $\gamma$ -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).

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

## 24 **Keywords:**

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

## 26 **1. Introduction**

27 Chiapas cream cheese (CCH) is a semi-ripened traditional cheese manufactured in the tropical south of  
28 Mexico. It is characterised by its acidic taste and creamy sensory properties (i.e. mouth feel). These  
29 characteristics are conferred during the process that involves 3-5 h of whole raw milk maturation  
30 followed by 2- 8 h of coagulation and acidification by endogenous lactic acid bacteria (LAB) at tropical  
31 conditions ( $>25^{\circ}C$ ) (González-Córdova et al., 2016). CCH was reported a humidity of 48%, a pH about  
32 4.0, and 5% of NaCl (Morales, Morales, Hernández, & Hernández-Sánchez, 2011). LAB are able to  
33 release bioactive compounds from milk proteins by proteolysis during cheese ripening. For instance,  
34 *Lactobacillus (Lb.) helveticus* DSM13137, a proteolytic cheese starter, releases the antihypertensive  
35 peptides Ile-Pro-Pro and Val-Pro-Pro during milk fermentation (Seppo, Jauhiainen, Poussa, & Korpela,  
36 2003). The ACE inhibitory activity (ACEi) has been attributed as one of the main antihypertensive  
37 mechanisms of bioactive peptides. ACEi was reported in ripened Red Cheddar and Camembert with an  
38  $IC_{50}$ , the amount of protein to inhibit the ACE activity by 50%, as low as 0.16 mg/mL, whereas non  
39 activity was detected in cottage, an unripen cheese (Okamoto et al., 1995). Furthermore, Gupta, Mann,  
40 Kumar & Sangwan (2013) showed a clear relationship between degree of hydrolysis (DH) and an  
41 increase of ACEi in cheese water-soluble fraction (WSF) when adding adjunct cultures at different  
42 stages of ripening Cheddar cheeses. Also, a positive correlation between ripening and the radical  
43 scavenging capability of Cheddar WSF was found when adding *Lb. casei* ssp *casei* 300 as adjunct  
44 culture (Gupta, Mann, Kumar, & Sangwan, 2009). Indeed, it was reported that CCH possess higher

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

## 61 **2. Material and methods**

### 62 **2.1 Reagents and cheese sample preparation and characterisation**

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

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

## 73 **2.2 Determination of total protein, degree of hydrolysis and peptide profile**

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

## 80 **2.3 Amino acid profile of cheese samples**

81 The free amino acid (FAA) profiles of cheese samples, including  $\gamma$ -aminobutyric acid (GABA), were  
82 examined using a derivatisation assay kit EZ-Faast (Phenomenex USA), and running on a GC- Agilent  
83 6890 GC-5975-MS system (Agilent, USA) in electron impact mode. The method used is based on  
84 Elmore, Koutsidis, Dodson, Mottram, & Wedzicha (2005). The results were compared to those found in  
85 raw milk.

## 86 **2.4 Isolation of bacteria**

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

## 95 **2.5 Genetic fingerprinting of bacterial isolates**

96 DNA was extracted from each isolate using the phenol/chloroform method and randomly amplified  
97 polymorphic DNA-PCR (RAPD-PCR) employed to estimate the genetic variations of the isolates. The  
98 reaction mixture (25  $\mu$ L) comprised 5  $\mu$ L of 5x GoTaq Flexi buffer (Promega, UK), 2.5  $\mu$ L of dNTPs  
99 (0.4 mmol/L of each of dATP, dCTP, dGTP, dTTP; Promega), 1.5  $\mu$ L of MgCl<sub>2</sub> (25 mmol/L; Promega),  
100 1  $\mu$ L of primer OPA-09 (5'-GGGTAACGCC-3'; 20 pmol/mL; Sigma Genosys, UK), 1  $\mu$ L of GoTaq  
101 DNA polymerase (1.5 U/  $\mu$ L; Promega), 1  $\mu$ L of template DNA (5 ng/ $\mu$ L) and 13  $\mu$ L of sterile water.  
102 PCR was performed using Prime thermal cycler (with heated lid, 100 °C; Techne) programmed for 40  
103 cycles of denaturation (30 sec at 94 °C), annealing (60 sec at 38 °C) and extension (2 min at 72 °C), with  
104 a final 10 min extension step (72 °C). The reaction products were separated via agarose gel  
105 electrophoresis (1.5% in 1x TAE buffer [Fisher, UK] containing ethidium bromide [0.5 ng/mL]), using  
106 1 Kb DNA ladder (Promega) as a molecular size indicator. DNA fragment patterns were visualized  
107 under UV light (Genesnap, Syngene) and analysed by Gel Compar II software.

## 108 **2.6 Fermentation of milk by isolated strains, diacetyl and catalase test**

109 Bacterial strains were reactivated on appropriate agar plates (aerobically/anaerobically), checked for  
110 purity (single colony type) and overnight brain heart infusion (BHI) broth cultures prepared (aerobically  
111 or anaerobically, as per original isolation). Milk fermentations were carried out in Hungate tubes with  
112 15 mL of 10% (w/v) reconstituted skim milk (RSM) using 1% inoculum of fresh overnight broth culture  
113 for each isolate (affording initial number of 10<sup>6</sup>-10<sup>7</sup> cells/mL). A negative control, using uninoculated  
114 BHI (1%) was also included. The fermentations were incubated at 30 °C with continuous agitation for  
115 24 h. Samples of 2 mL were then taken and heated to 72 °C for 1 min to stop the enzymatic proteolysis  
116 and then centrifuged at 12,000 g for 10 min. The supernatant was filtered and stored at -20 °C until  
117 further analysis.

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

## 121 **2.7 Activity *in vitro* assays**

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

#### 125 *ACE inhibitory activity*

126 The ACEi of cheese samples was determined using N-[3-(2-furyl)acryloyl]-Phe-Gly-Gly (FAPGG)  
127 according to the method described by Henda et al. (2013) with some modifications. Briefly, in a  
128 microplate well 10  $\mu$ L of ACE (250 mU solution with 0.05 M Tris, 0.3 M NaCl in 50% glycerol  
129 solution, with the pH adjusted to 7.5 with 5 M HCl) was mixed with 150  $\mu$ L of 0.88 mmol/L FAPGG in  
130 0.05 M Tris, 0.3 M NaCl and 10  $\mu$ L of test sample (diluted cheese or fermented milk fraction). 0.05 M  
131 Tris, 0.3 M NaCl buffer was used as negative control and 5 M HCl was used as positive control  
132 (standard inhibition). The reaction kinetics was followed in a Tecan Microplate Reader – A-5082  
133 Spectra FLUOR plus (Austria) for 30 minutes to obtain the slope inhibitor [FAPGG] vs time (min). The  
134 ACEi% was calculated in relation to the slope generated when no inhibitor was present in the reaction  
135 (slope blank) according to equation 1 (below).

136

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

138

139 The IC<sub>50</sub>, defined as the concentration of protein needed to inhibit the activity of the enzyme by half,  
140 was calculated as the concentration needed to reduce the slope by 50% in relation to the slope blank.

141 The ACEi% in fermented milk fractions with selected strains, as described above, was determined  
142 according to the HPLC method described by Gonzalez-Gonzalez et al. (2011) using Hip-His-Leu as  
143 substrate.

#### 144 *Antioxidant activity de FRAP*

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

148

149 *Antioxidant activity by ABTS+• radical*

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

156

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

158

159 Where ABS<sub>control</sub> is the absorbance of ABTS<sup>++</sup> in PBS and ABS<sub>sample</sub> is the reaction absorbance with the sample.

160

161

## 162 **2.8 Data analysis and statistics**

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

## 165 **3. Results and Discussion**

### 166 **3.1 Proteolysis in Chiapas cheese**

167 The proteolytic activity undergoing in CCH samples is depicted in the peptide profile chromatograms.  
168 Fig. 1 showed intact milk proteins in RSM as control (Fig. 1A), and a significant breakdown in AVCH  
169 (Fig. 1B) and HTCH (Fig. 1C) peptides profiles. Larger peak areas of peptides in AVCH suggests  
170 higher proteolytic activity than in HTCH. This is confirmed by the FAA profiles, which show higher  
171 concentrations of total FAA in AVCH (3.89 g/kg) than in HTCH (0.37 g/kg) (Table 2). These  
172 concentrations are similar to those found in Spanish cheese (0.19 and 69 g/kg) (Diana, Rafecas, Arco, &  
173 Quílez, 2014). Moreover, the essential amino acids (AA) leucine, phenylalanine, lysine and valine were  
174 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, & Vuilleumard (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<sub>50</sub> 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<sub>50</sub> 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* subsp *casei* IC<sub>50</sub> 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  $IER \approx 9$ . 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% ( $>1600 \mu\text{mol/L TEAC}$ ),  
245 is higher than that found on sweet whey (36%) and  $\beta$ -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  $860 \mu\text{mol/L TEAC}$  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  $311.66 \mu\text{mol/L TEAC}$  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 ( $2138.95 \pm 20.23 \mu\text{mol/L}$   
254  $\text{TEAC}$ ), followed by strain s6 ( $2059.06 \pm 26.27 \mu\text{mol/L TEAC}$ ) after 48 hours of fermentation are  
255 higher than those previously reported. Overall, the three selected isolates from CCH (s6-HTCH, s10-

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

#### 258 **4. CONCLUSIONS**

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

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277 no conflict of interest.

278

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**Table 1.** IC<sub>50</sub> values for ACE inhibitory activity, protein content and pH for Chiapas cheese samples from Veracruz (AVCH) and Tabasco (HTCH) (n=2, ± s.e.).

<b>Activity</b>	<b>AVCH</b>	<b>HTCH</b>
ACE inhibition (IC <sub>50</sub> mg/mL)	2.75 ± 0.50 <sup>a</sup>	1.75 ± 0.49 <sup>a</sup>
Total protein (g/100g)	26.95 ± 3.44 <sup>b</sup>	23.72 ± 2.88 <sup>b</sup>
pH	3.90 ± 0.06 <sup>c</sup>	3.95 ± 0.09 <sup>c</sup>

<sup>a,b,c</sup> No statistical difference between the means with the same letter;  $\alpha=0.05$ .

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

<b>Amino acid</b>	<b>Raw milk g/kg</b>	<b>AVCH g/kg</b>	<b>HTCH g/kg</b>
Alanine	0.005	0.283	0.072
$\alpha$ -aminobutyric acid	0.001	0.022	ND
Asparagine	Tr <sup>1</sup>	0.135	0.009
Aspartic acid	0.010	0.148	0.010
<b><math>\gamma</math>-aminobutyric acid</b>	<b>ND<sup>2</sup></b>	<b>0.784</b>	<b>0.160</b>
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

<sup>2</sup>Tr, traces

<sup>1</sup>ND, None detected.

**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).<sup>†</sup>

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

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† (-) negative reaction; (+) positive reaction. pH of uninoculated RSM (control) was  $6.50 \pm 0.03$ . 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%.



1

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

5

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

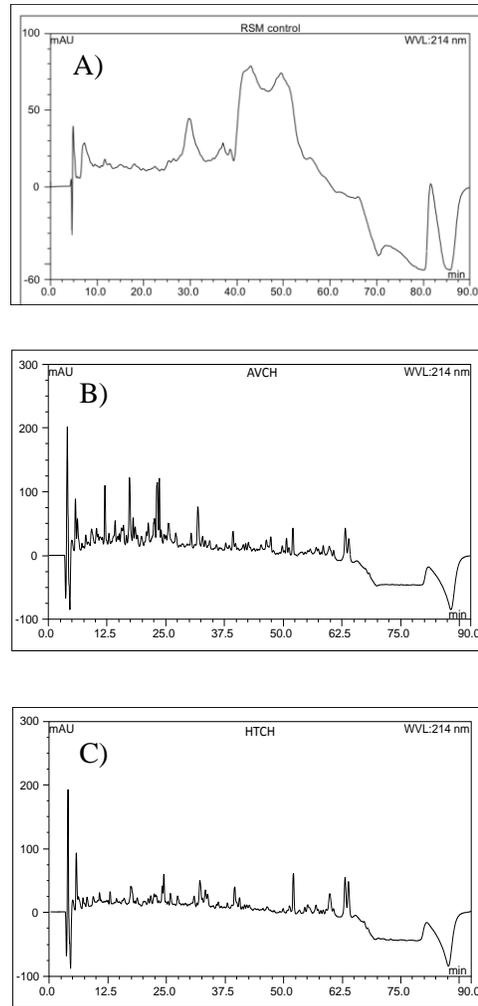
6 \* Treatments with the same letter are not significantly different, Tukey HSD test:  $\alpha=0.05$

7

8

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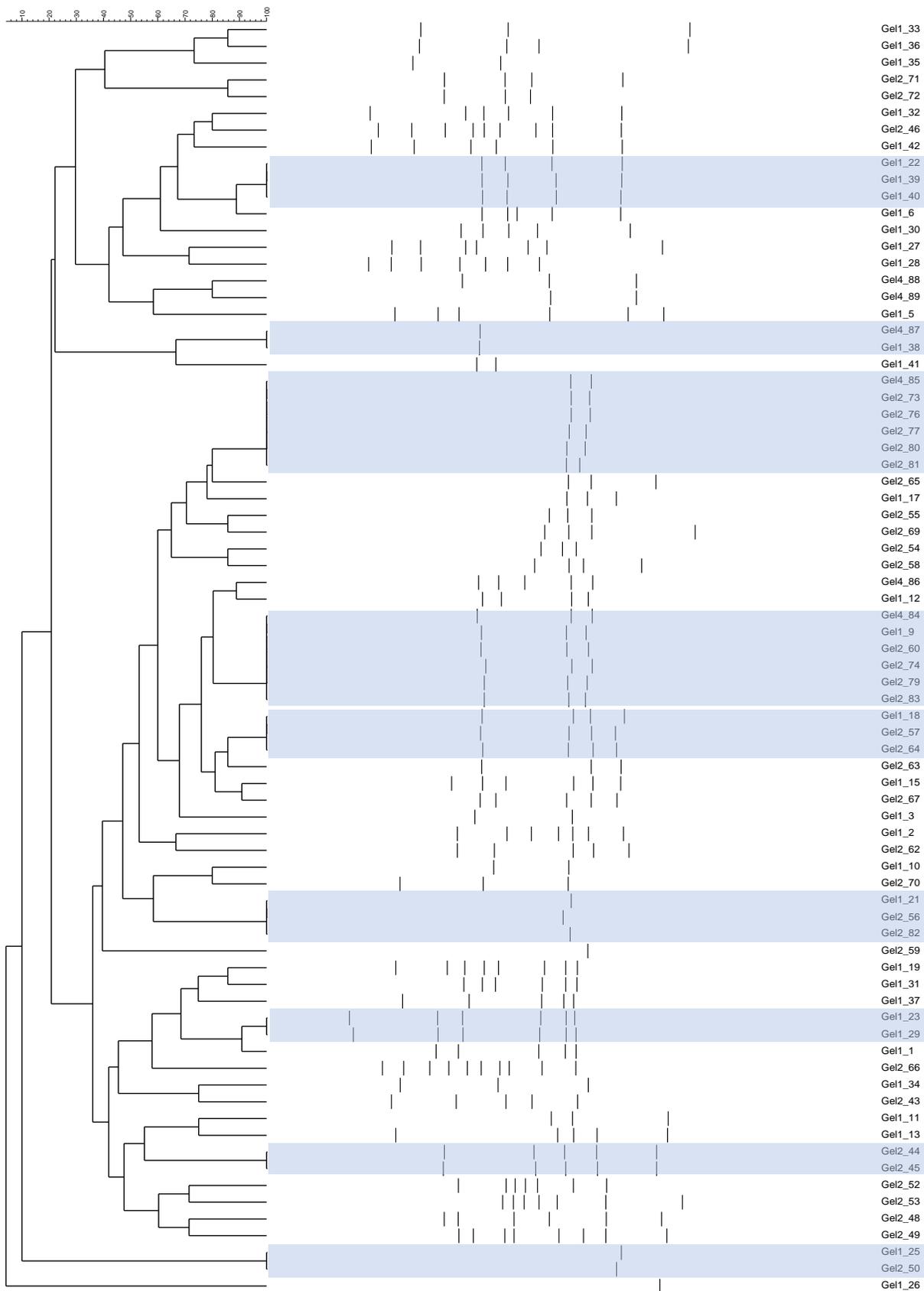
10



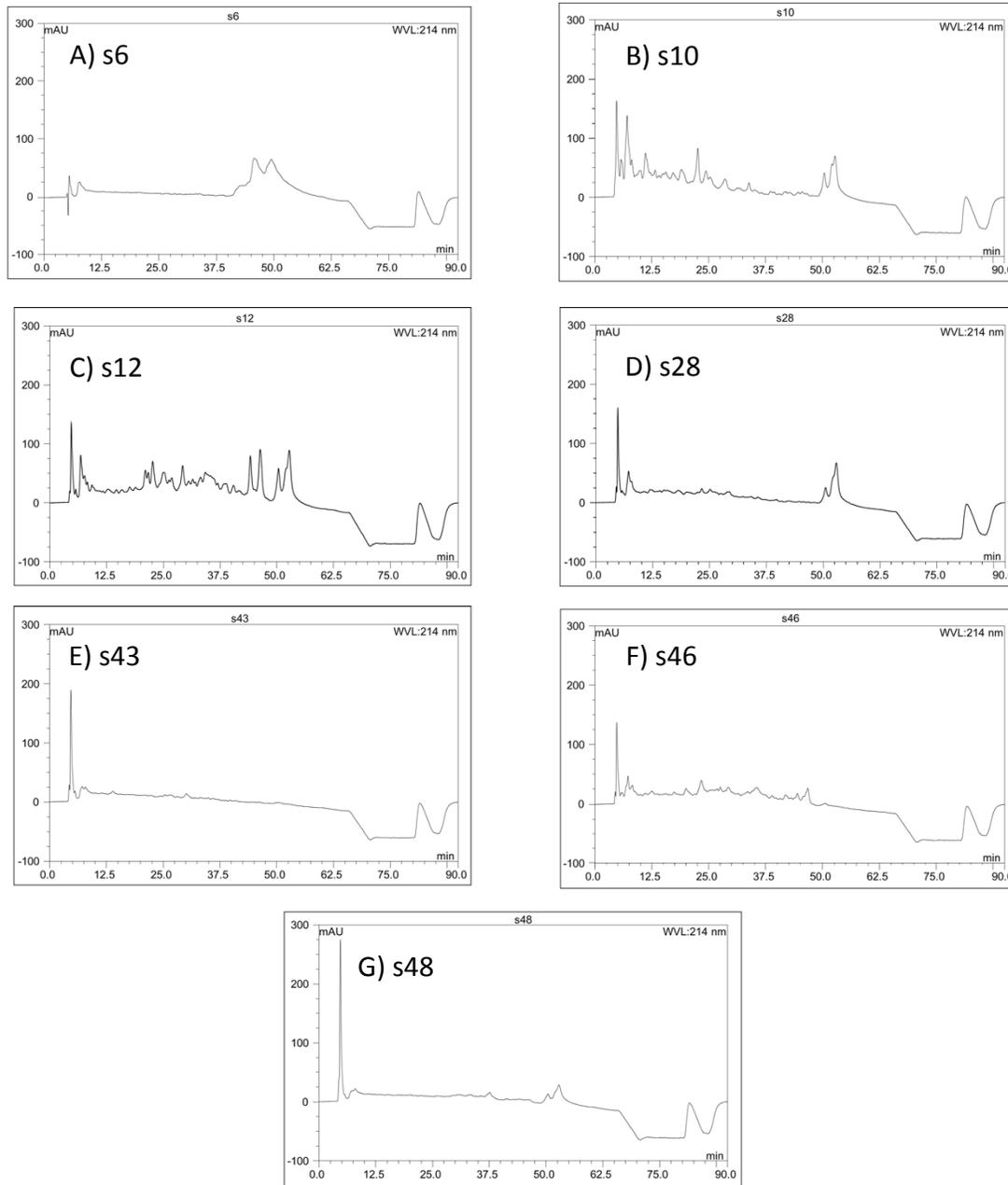
**Fig. 1.** RP-HPLC peptide profiles of A) reconstitute skimmed milk (RSM); B) Chiapas cheese from Veracruz (AVCH); and C) from Tabasco (HTCH).

RAPD

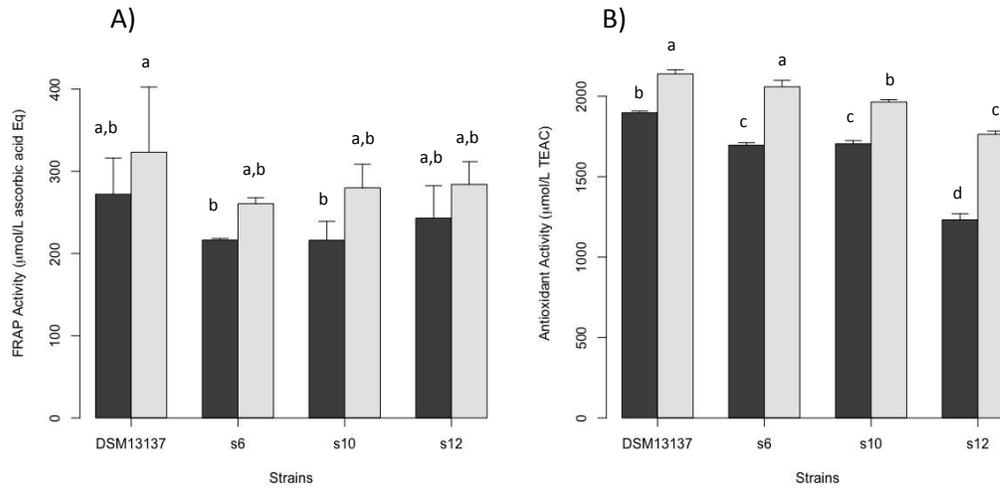
RAPD



**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).



**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.



**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  $\pm$  SD (n=3). Treatments with the same letter are not significantly different, Tukey HSD test:  $\alpha=0.05$