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## Food & Function

### **Antioxidant, ACE-inhibitory and antimicrobial activity of fermented goat milk: activity and physicochemical properties relationship of the peptide components**

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18 **Abstract**

19 Increasing evidence on goat milk and their derived products health benefits beyond their  
20 nutritional value show their potential as functional foods. In this study, goat milks' fractions were  
21 tested for their total antioxidant capacity measured by different methods (ORAC, ABTS, DPPH  
22 and FRAP), as well as the angiotensin-I-converting-enzyme inhibitory and antimicrobial (against  
23 *Escherichia coli* and *Micrococcus luteus*) activities. Different whey fractions (whey; cation  
24 exchange membrane permeate, P and retentate, R) of two fermented skimmed goat milks  
25 (ultrafiltered goat milk fermented with the classical starter bacteria or with classical starter plus  
26 the *Lactobacillus plantarum* C4 probiotic strain) were assessed. Additionally, P fractions were  
27 divided into two sub-fractions after passing them through a 3 kDa cut-off membrane: (a) the  
28 permeate with peptides <3 kDa (P<3); (b) and the retentate with peptides and proteins >3 kDa  
29 (P>3). No differences in biological activities were observed between the two fermented milks.  
30 However, the biological peptides present in the P<3 fraction showed the highest total antioxidant  
31 capacity (for the ORAC assay) and angiotensin-I-converting-enzyme inhibitory activities. Those  
32 present in the R fraction showed the highest total antioxidant capacity against ABTS<sup>•+</sup> and DPPH<sup>•</sup>  
33 radicals. Some antimicrobial activity against *E. coli* was observed for the fermented milk with the  
34 probiotic, which could be due to some peptides released by the probiotic strain. In conclusion,  
35 small and non basic bioactive peptides could be responsible of most of angiotensin-I-converting-  
36 enzyme inhibitory and antioxidant activities. These findings reinforce the potential benefits of the  
37 consumption of fermented goat milk in the prevention of cardiovascular diseases associated to  
38 oxidative stress and hypertension.

39

40 **Keywords:** goat milk, antioxidant, antimicrobial, antihypertensive, ultrafiltration, ion exchange

41

42

## 43 **Introduction**

44 Fermented milks satisfy daily nutritional requirements for several nutrients and exert different  
45 health benefits.<sup>1</sup> Furthermore, it is an important source of many bacterial strains owing to the  
46 appropriate compatibility among some of them.<sup>2</sup> Fermented milks contain several probiotic strains,  
47 which additionally increase the already known benefits of these dairy products. Milk fermentation  
48 by classical starter bacteria (St) (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus*  
49 *salivarius* subsp. *thermophilus*) changes milk properties and increases its digestibility by a  
50 decrease in lactose concentration and pH. This process could also release biological active peptides  
51 from their inactive forms present in the corresponding sequence of the precursor protein. The  
52 specific sequence and length of released peptides depend on two main factors: (a) the precursor  
53 protein, which is different in sequence depending on the animal specie and even on the breed;<sup>3</sup> (b)  
54 the starter bacteria, since the proteolytic system is inherent to each bacteria strain. The healthy  
55 benefits of these bioactive peptides may be attributed to their demonstrated antimicrobial,  
56 antioxidant, antihypertensive, antithrombotic, immunomodulatory and opioid activities.<sup>4</sup> Many of  
57 the bioactive peptides have demonstrated to have multi-functional properties. Nevertheless, their  
58 specific activity depends on the amino acid composition as well as sequence. In this sense, it is  
59 well known that anionic peptides do not affect gram-negative bacteria because of repulsive  
60 electrostatic interactions between the negatively charged outer membrane and the anionic peptides.<sup>5</sup>  
61 On the other hand, some cationic peptides have shown antimicrobial effect against gram-negative  
62 bacteria. However, not all the positively charged peptides exert antimicrobial activity and the  
63 action mechanism of milk-derived antimicrobial peptides remains uncertain.<sup>6</sup> In any case, several  
64 peptides have been discovered with antimicrobial activity that can find industrial application.<sup>6</sup>

65       Among the different functions of bioactive peptides, antioxidant properties are very important  
66 because high levels of reactive oxygen species (ROS) and free radicals in the organism are  
67 associated to several diseases like cancer, diabetes, cardiovascular diseases, arthritis, allergies as

68 well as to aging.<sup>7</sup> In addition, ROS presence in food causes quality deterioration and shelf life  
69 reduction by lipid oxidation.<sup>3</sup> It is known that the defense systems of organisms are often not  
70 enough to prevent oxidative damage. Some researchers have stated that antioxidant peptides  
71 present in the food system play a vital role in the maintenance of antioxidant defense systems in  
72 the organism by preventing the formation of free radicals or by scavenging free radicals and  
73 reactive oxygen species, and Cheng et al. even recommended their supplementation.<sup>7</sup> An  
74 increasing number of food protein hydrolysates and peptides have been found to exhibit  
75 antioxidant activity, especially in peptides produced from bovine milk casein.<sup>3</sup> *In vitro*  
76 measurement of antioxidant activity is key in the evaluation of the antioxidant potential of  
77 bioactive peptide-enriched preparations. Due to the complex nature of antioxidants, there is no a  
78 single technique to measure the total antioxidant capacity (TAC) of a food system. Therefore, a  
79 variety of analytical techniques are employed with this aim, which can roughly be classified into  
80 two types namely, the assays based on hydrogen atom transfer (HAT) reactions and those based  
81 on electron transfer (ET).<sup>8</sup> Then, to study the antioxidant activity of any sample it is necessary to  
82 use at least one assay of each type in order to obtain a more complete evaluation of the TAC as the  
83 different mechanisms of antioxidant action will be taken into account;<sup>9</sup> this is particularly  
84 important when multicomponent samples are being evaluated.

85 Most of biologically active peptides generated from milk proteins have demonstrated an  
86 angiotensin-I-converting enzyme-inhibitory activity (ACEi).<sup>10</sup> This effect leads to a decrease in  
87 angiotensin II (potent vasoconstrictor) and a concomitant increase in the bradykinin level, finally  
88 yielding an overall reduction in the blood pressure.<sup>11</sup> Although the inhibitory capacity of milk  
89 derived peptides is lower than that of chemically designed drugs, their production from natural  
90 sources could represent a healthier and more natural alternative for chronic treatment, without the  
91 side-effects associated to antihypertensive drugs.<sup>11</sup> It is known that most publications on ACEi and

92 antihypertensive peptides consider peptides obtained from cow milk.<sup>4</sup> However, in recent years  
93 goat milk proteins have become an important alternative source of ACEi bioactive peptides.<sup>12</sup>

94 Previous *in vitro* studies have demonstrated that the probiotic strain *L. plantarum* C4 has a  
95 positive influence in a range of biological functions such as, mineral bioavailability,<sup>13</sup> modulation  
96 of the intestinal microbiota<sup>14</sup> and protective and immunomodulatory capacity in a murine model  
97 of yerseniosis.<sup>15</sup> Taking into consideration all previous findings, it was hypothesised here that the  
98 probiotic strain could also enhance the antioxidant, ACE-inhibitory and antimicrobial activities,  
99 in fermented goats' milks.

100 Only a few studies have focused on the bioactivity of fermented goat milk peptidic fractions.  
101 Therefore, the aim of the present study was the evaluation of the biological activities (antimicrobial  
102 activity against *Escherichia coli* and *Micrococcus luteus*, TAC measured by ORAC, ABTS, DPPH  
103 and FRAP methods, and ACEi-activity) of two fermented skimmed goat milks fermented with the  
104 classical starter bacteria [StFM] or with classical starter plus the *Lactobacillus plantarum* C4  
105 probiotic strain [St+LPFM]). The use of the probiotic strain *L. plantarum* C4 on the milk protein  
106 concentrates produced by a local breed of goat for the fermentation process was investigated here  
107 for the first time in order to produce a milk product with enhanced biological activities. In addition  
108 a novel approach was followed for the physicochemical characterisation (size and charge) of the  
109 peptides in the fermented milk in relation to their bioactivities.

110

## 111 **Results and discussion**

### 112 **Total protein analysis**

113 As stated in Table 1 a significantly higher protein concentration was observed in whey and  
114 permeate (P) fractions when compared to the retentate (R), which means a large proportion of the  
115 peptides produced by the tested fermenting strains were anionic or nonionic. Additionally, the

116 fractions of StFM have a higher protein concentration than St+LPFM; that may be due to  
117 differences in the fermentation process between St and *L. plantarum* C4, in particular pH, as a  
118 lower pH was recorded for the fermentation with the probiotic ( $4.25 \pm 0.02$ ) vs. StFM ( $4.39 \pm 0.05$ )  
119 which could have led to more protein coagulation and less soluble protein/peptide.<sup>16</sup>

120

### 121 **Total antioxidant capacity**

122 The results obtained for TAC showed a good correlation with protein content ( $p < 0.001$ ; r:  
123 ORAC=0.772, ABTS=0.906 and FRAP=0.950), which could be attributed to the activity of  
124 peptides present in those fractions. In order to find which of the fractions had the most active  
125 peptides the results were also expressed as  $\mu\text{mol Trolox equivalents mg of protein}^{-1}$  (Fig. 1). The  
126 most active fractions were different to those identified when expressed as Trolox equivalents  $\text{mL}^{-1}$   
127 <sup>1</sup>, which means that not always the most active peptides were in the most active fractions.

128 The highest TAC of the fermented milk fractions (Fig. 1) was measured by ORAC for the P<3  
129 fraction (reaching  $2.927 \pm 0.043 \mu\text{mol Trolox equivalents mL}^{-1}$  in the StFM). However, according  
130 to the other assays, the different milk fractions did not reach  $0.4 \mu\text{mol Trolox equivalents mL}^{-1}$   
131 (Fig. 1) for any of the fermented milks (StFM and St+LPFM). Thus, in the case of the FRAP and  
132 ABTS assays, the highest TAC was found for the whey and P fractions. Therefore these results  
133 show that fractionation by IEX did not result in increased activity as whey and P samples had  
134 similar TAC according to all methods while the retained fraction had lower activity (particularly  
135 according to ORAC and FRAP methods). On the other hand the fractionation by size  
136 (ultrafiltration) resulted in significant differences in antioxidant capacity (Fig. 1) with an important  
137 increase in activity. P<3 kDa fractionation showed higher values according to ORAC, ABTS and  
138 DPPH methods, while no significant differences were observed between these fractions in FRAP  
139 assay.

140 The measured TAC (by ORAC and ABTS assays) for almost all analyzed fractions was  
141 significantly higher for StFM than for St+LPFM (Fig. 1). Only the samples from St+LPFM had  
142 significantly higher antioxidant capacity in whey fraction according to DPPH assay. The variation  
143 in TAC when using the different methods could be attributed to the presence of different peptides  
144 that act by different mechanisms. It has been demonstrated that the TAC of dairy products is  
145 mainly due to the activity of peptides. Some authors agreed that the main contribution to TAC  
146 comes from casein fractions in milk, suggesting that such effect is related to the self-oxidation of  
147 caseins' amino-acid residues as well as their derived peptides. Additionally, they reported that this  
148 activity cannot be replaced by free amino acids since it is the primary structure of casein itself who  
149 plays a determining role.<sup>17</sup> Among the caseins that release antioxidant peptides,  $\beta$ -CN could be  
150 preferably degraded by lactic acid bacteria because it is more unstructured and accessible to  
151 cleavage, and therefore hydrolyzed to a greater extent.<sup>7</sup> On the other hand,  $\beta$ -LG and lactoferrin  
152 have been reported as key components for their high scavenging activity, releasing also peptides  
153 with this activity.<sup>18</sup> The TAC of peptides has been described as remarkably dependent on factors  
154 like molecular weight, amino acid composition and sequence.<sup>19</sup> Many authors reported that most  
155 of milk protein-derived peptides with antioxidant activity have less than 20 amino-acid  
156 residues.<sup>1,7,11</sup> This is in agreement with our results as the P<3 fraction, with peptides of MW<  
157 3000 (up to about 20 amino-acid residues), had the highest TAC (measured by ORAC), reaching  
158 more than 1  $\mu$ mol trolox equivalents mg protein<sup>-1</sup> (Fig. 1). Nevertheless, Virtanen et al.,<sup>20</sup> reported  
159 the contrary, supporting higher scavenging activity against the ABTS<sup>•+</sup> radical of peptides with  
160 more than 4 kDa. However, we found that the R fraction contained the peptides with significantly  
161 highest TAC against ABTS<sup>•+</sup> and DPPH<sup>•</sup> radicals (~ 0.4  $\mu$ mol trolox equivalents mg protein<sup>-1</sup>;  
162 Fig. 1). These findings agree with the results reported by other researchers,<sup>21</sup> who stated that basic  
163 peptides had greater capacity to scavenge hydroxyl radical than weak acidic or neutral ones.

164 Few studies have indicated that the radical scavenging activity is strain-specific and that the  
165 higher proteolysis is not always associated with higher TAC.<sup>20,22</sup> In our study no significant

166 differences were observed for P<3 fraction ( $\mu\text{mol trolox equivalents mL}^{-1}$ ) between StFM and  
167 St+LPFM, and for almost any other fraction when results were expressed as  $\mu\text{mol trolox}$   
168  $\text{equivalents mg of protein}^{-1}$ . Therefore, the putative probiotic strain *L. plantarum* C4 by itself or  
169 by its interaction with St produced no increase in the antioxidant capacity of the fractions.

170 It is known that goat milk has more  $\beta$ -CN than cow milk. In particular, the analyzed fermented  
171 goat milks were concentrated in caseins, therefore it was expected to obtain more  $\beta$ -CN derived  
172 peptides than from cow fermented milk. Notwithstanding, results were in the range of those  
173 reported for whey fractions of cow fermented milks tested against ABTS, ranging from 0.2774 to  
174 2.0356  $\mu\text{mol trolox equivalents mL}^{-1}$ .<sup>22</sup> However, the whey fraction had higher TAC than those  
175 reported for nonfermented milks (0.489 in UHT and 1.078  $\mu\text{mol trolox equivalents mL}^{-1}$  in  
176 pasteurized milk).<sup>23</sup> This finding is probably related to the proteolytic activity of the fermenting  
177 strains, which were able to release the antioxidant peptides from milk proteins.<sup>24</sup>

178 On the other hand, StFM and St+LPFM were produced only in 6 h whereas some authors  
179 reported that TAC increases with fermentation time up to 24-48 h.<sup>7,22</sup> Some studies reported low  
180 TAC of the whey fraction, but after fractionation by HPLC, different fractions with higher TAC  
181 were obtained.<sup>22</sup> Consequently, future research should focus on fractionating and identifying the  
182 peptides responsible of the TAC in the whey fraction.

183 Saura-Calixto and Goñi<sup>24</sup> reported a total antioxidant daily intake in a typical Spanish diet of  
184 3,549  $\mu\text{mol trolox equivalents (ABTS)}$  and 6,014  $\mu\text{mol trolox equivalents (FRAP)}$ . Taking into  
185 account the whey obtained from a portion of fermented milk sample (200 g), the percentage for  
186 which this whey participate in the daily antioxidant intake is 0.75% for the ABTS and 0.50% for  
187 the FRAP methods.<sup>24</sup> However, the total antioxidant activity of the fermented milk should be  
188 higher if we consider the precipitated fraction, with precipitated caseins and bacteria for which an  
189 antioxidant activity has also been reported elsewhere.<sup>1</sup>

190 Finally, the TAC (Trolox equivalents  $\text{mL}^{-1}$ ) values of the fractions obtained by the different  
191 methods were significantly ( $p < 0.001$ ) correlated with each other ( $r > 0.830$  and  $r = 0.770$  for the

192 ABTS-FRAP and ORAC-FRAP, respectively). DPPH was not significantly correlated with any of  
193 the other methods. However, when the TAC was expressed based on protein content a significant  
194 correlation was also found for DPPH-ABTS ( $r= 0.937$  at  $p < 0.001$ ) and ORAC-FRAP ( $r= 0.807$   
195 at  $p < 0.001$ ). This additional significant correlation between DPPH-ABTS could be explained by  
196 considering mainly the peptides/proteins responsible for the antioxidant capacity. This is very  
197 interesting as there was very good correlation between methods testing antioxidant capacity based  
198 on the same mechanism, as DPPH and ABTS are based on both hydrogen atom transfer (HAT)  
199 and single electron transfer reactions (SET); the highest TAC was found in the retentate according  
200 to the ABTS and DPPH methods. Moreover there was also good correlation between methods  
201 based on different mechanisms FRAP (SET) and ORAC (HAT) but with biological relevance  
202 ; the highest TAC was found in permeate according to the FRAP and ORAC methods. These  
203 results demonstrate that different types of antioxidants are recovered in the different fractions with  
204 differences in their antioxidant mechanism.

205

#### 206 **ACEi% activity**

207 Firstly, the measured  $IC_{50}$  obtained for captopril was  $0.023 \mu\text{M}$ , in the range reported by the  
208 manufacturer ( $0.021 \pm 0.013 \mu\text{M}$ ). This result confirms the reliability of the method used. In Fig.  
209 2a, the ACEi activities of the different fractions of fermented goat milks expressed as percentage  
210 of inhibition are shown. The whey and P<3 fractions had the highest ACEi activity (about 50%).  
211 Interestingly the R fraction did not show any activity.

212

213 Given that in previous *in vitro* studies<sup>13-15</sup> the fermentation by the probiotic strain *L. plantarum*  
214 C4 had led to a range of biological functions the ACEi activity was tested here. Nevertheless, no  
215 significant differences were found between StFM and St+LPFM for any of the analysed fractions.  
216 Therefore, adding the *L. plantarum* C4 probiotic strain did not significantly increase the ACEi

217 when compared to StFM. Gonzalez-Gonzalez et al.<sup>27</sup> found a strain of *L. plantarum* able to produce  
218 a supernatant with high ACEi activity after 24 h of fermentation. Regarding the other  
219 microorganisms used, *L. bulgaricus* has been reported as one of the most proteolytic  
220 microorganism as well as a great producer of ACEi peptides<sup>25</sup>; high ACEi activity (more than  
221 50%) was measured in supernatants obtained from milk fermented with 4 strains of *L. bulgaricus*  
222 <sup>26</sup>. As stated above for TAC, ACEi activity was significantly correlated with protein concentration  
223 ( $r^2=0.800$ ;  $p < 0.001$ ). When results were expressed as ACEi% mg protein<sup>-1</sup>, the permeate fractions  
224 had the highest activity and in particular the P <3 fraction (Fig. 2b). Therefore, as expected, smaller  
225 peptides had the highest ACEi (Fig. 2b). In that sense, the fractionation by size led to an increase  
226 in the activity. Interestingly charge had also an effect on activity<sup>28</sup> as the positively charged  
227 fraction of peptides (R) had very little activity (Fig. 2b). Hence the basic peptides had much less  
228 activity than the acidic (negatively charged and noncharged) peptides. This is in accordance with  
229 the results of Welderufael et al.,<sup>28</sup> who found that one of the fractions of the enzymatic whey  
230 hydrolysate with peptides derived from  $\beta$ -lactoglobulin with highest ACEi and lowest IC<sub>50</sub>,  
231 contained as main peptides acidic peptides such as IIAE with isoelectric point 4.6.

232 ACEi% reported values for fermented milk whey are very variable depending on the strain  
233 used. For milks fermented with *L. bulgaricus* and *S. thermophilus*, most of the reported values are  
234 around the 50%, ranging from 25% to 70% of ACEi% activity<sup>11,25</sup>. Some work was carried out  
235 with 13 strains at 3 different final pH's and found that the maximum inhibitory activity was 51%  
236 for milk fermented with *Lactococcus lactis* 3906 and with final pH 4.3. However, the milk  
237 fermented with *S. thermophilus* did not reach the 18% of ACEi activity.<sup>29</sup> Otte et al. demonstrated  
238 a negative correlation between pH and ACEi activity of milk fermented with two strains of *L.*  
239 *helveticus* and two species of the *Lactococcus* genus, reporting a range from 8 % to 50% of ACEi  
240 activity.<sup>30</sup> However, higher values of ACEi activity were found in milk fermented with other

241 strains like Kumis bacteria, ranging from 10.1 to 74.3 % and up to 100% when fermented with St  
242 plus *L. acidophilus* L10, *L. casei* L26 and *B. lactis* B94<sup>11,31</sup> .

243 On the other hand, the ACEi activity has been demonstrated to be related to ionic calcium  
244 ( $\text{Ca}^{2+}$ ), since its concentration may activate or inhibit the ACE.<sup>27</sup> We demonstrated that goat UFM  
245 was concentrated in caseins and that the ultrafiltration process changed  $\text{Ca}^{2+}$  distribution  
246 [percentage of Ca associated to caseins changed from 63% in goat raw milk (RM) to 51% in goat  
247 UFM] and  $\text{Ca}^{2+}$  content from  $135.2 \pm 10$  to  $165.6 \pm 15.1$  mg/100g in goat RM and UFM,  
248 respectively.<sup>32</sup> Additionally, the most potent antihypertensive and ACE-inhibitory peptides are  
249 generated from caseinates and casein fractions.<sup>33</sup> These findings could explain the high ACEi %  
250 found in our fermented goat milk samples. Moreover the fermentation with the probiotic *L.*  
251 *plantarum* did not result in increased ACEi activity. One of its strains was reported to be the best  
252  $\gamma$ -amino butyric acid (GABA) synthesizer; GABA is a non-protein derived amino acid with  
253 demonstrated hypotensive effect in rats and humans.<sup>34</sup> Future studies should focus on GABA  
254 production by the probiotic *L. plantarum* C4, due to its possible relationship with the hypertension  
255 control.

256  
257  
258

### **Antimicrobial activity**

259 According to the well diffusion assay, no antimicrobial activity of the supernatants against *E.*  
260 *coli* was observed ( $p > 0.05$ ). By contrast, in the whey and P fractions, *E. coli* grew even better  
261 than in the control assay. Nevertheless, in the spot assay for both whey and P fractions *E.coli* did  
262 not grow where the drop was placed, probably due to the low pH of the samples (4.33 and 4.59 for  
263 whey and P fractions, respectively). However, R fraction, with higher pH (6.97) due to the  
264 presence of cationic peptides did not show any activity against *E. coli*. In relation to *M. luteus*, we  
265 did not find any inhibition neither in the well diffusion assay nor in the spot test. On the contrary,  
266 even higher growth was found around the well of the whey fraction compared to the other fractions

267 where no effect was shown. Additionally, the co-culture assay was carried out to evaluate more  
268 precisely the possible inhibition of *E. coli* by the studied fractions. None of the fractions of the  
269 fermented milk studied showed antimicrobial activity and the pathogen grew almost as much as in  
270 the control (Fig. 3). However, after 24 h significant differences in *E. coli* viable bacteria among  
271 control and whey and P fractions of both fermented milks (StFM and St+LPFM), and R fraction  
272 of St+LPFM, were found. This inhibition could be due to the acidic pH of whey and P fractions  
273 (as mentioned above). However, the R fraction had a pH more similar to the control's. So in this  
274 case, the antimicrobial activity could be due to the cationic peptides isolated in this fraction, such  
275 as caprine lactoferricin, which has been shown antibacterial activity against *E. coli*<sup>35</sup>. Ionic charge  
276 is crucial for the attachment of peptides to the bacterial membrane<sup>5</sup>; we had hypothesised that  
277 cationic peptides would have higher activity than anionic or non charged peptides however, our  
278 results did not agree with this. The mechanism of action of milk-derived antimicrobial peptides  
279 remains uncertain and other physicochemical properties such as size amphiphilicity and  
280 conformation may play a role in their interaction with bacterial membranes.

## 281 **Experimental**

### 282 **Samples**

283 Goat milk samples from the Murciano-Granadina local breed were obtained from local farms  
284 (Granada province, Southeastern Spain). Specifically, every week along five weeks five batches  
285 with five samples for StFM and for St+LPFM were done, according to a previously standardised  
286 procedure.<sup>32</sup> Each individual sample was analysed by triplicate.

287

### 288 **Sample fractionation**

289 Fermented milk samples were fractioned in three steps (Fig. 4). In *the first step* the *whey fraction*  
290 was obtained. All samples were centrifuged at 3000g and 4 °C for 30 min (Sigma 2-16PK,

291 Sartorius, Goettingen, Germany). Then, the supernatant was separated, freeze-dried and stored  
292 under refrigeration and nitrogen atmosphere until analysis. Before the fractionation, freeze-dried  
293 samples were dissolved in water up to the initial volume and then filtered through 0.22 µm size  
294 pore filters Millex® - GS (Merck Millipore Ltd., Cork, Ireland) in a laminar flow cabinet and  
295 stored in sterile containers.

296 In the *second step a cation exchange* was applied. Sartobind filter MA-15 Units (Sartorius,  
297 Goettingen, Germany), with a strong acidic cation exchanger membrane. The procedure was  
298 carried out according to the operating instructions following four steps: (a) equilibration with 10  
299 mL of 10mM potassium phosphate buffer at pH 4.5; (b) loading with 5 mL of sample; (c) washing  
300 with 10 mL of equilibration buffer; (d) and finally elution with 5 mL of elution buffer  
301 (equilibration buffer + 1 M NaCl at pH 4.5). Then, the cation exchange units were cleaned with  
302 0.2 N NaOH for 30 min and equilibrated with 10 mL of equilibration buffer. All steps were  
303 conducted at 3 drops/s. With this method, two fractions for each sample were obtained: (1)  
304 *Permeate* (P) composed by anionic or zwitterions peptides and proteins at pH 4.5 that permeates  
305 when loading the sample; (2) and *Retentate* (R) composed by cationic peptides and proteins at pH  
306 4.5 retained in the resin and extracted in the elution step. We will refer to them as peptides because  
307 we assume that both fractions (P and R) could have bioactivity.

308 In the *third step ultrafiltration* was applied; molecules will be separated according to size only  
309 by a membrane with molecular weight cut off (MWCO) of 3 KDa. (Vivaspin20, Sartorius,  
310 Goettingen, Germany), The ion exchange permeates were fractionated into: (1) Permeate ( P<3 )  
311 which contained compounds sized less than 3 kDa anionic or zwitterions peptides; (2) and retentate  
312 (P>3) which contained compounds sized more than 3 kDa anionic or zwitterions peptides and  
313 proteins. As stated above, we will refer to them as peptides.

314

315 **Total soluble protein content**

316 The total protein content of the samples was determined based on the bicinchonic acid (BCA)  
317 assay according to the previously optimized method.<sup>36</sup> The absorbance was measured at 562 nm  
318 within 10 min using an Ultrospec 1100 pro UV/Visible spectrophotometer (Amersham  
319 Biosciences, Little Chalfont, UK). Serial dilutions of bovine serum albumin (Sigma-Aldrich,  
320 Steinheim, Germany) were used as standard and bidistilled water as blank.

321

### 322 **Total antioxidant capacity (TAC) measured by ORAC, ABTS, DPPH and FRAP assays**

323 The *TAC* using the *oxygen radical antioxidant capacity assay (ORAC)* was determined according  
324 to the method described by Huang et al.<sup>37</sup> slightly modified. In the *ABTS assay*, the antioxidant  
325 capacity was estimated in terms of radical scavenging activity following the procedure described  
326 by Pellegrini et al.<sup>38</sup> In the *DPPH assay*, the antiradical activity of different samples was estimated  
327 according to the procedure reported by Brand-Williams et al.,<sup>39</sup> which was adapted to a microplate  
328 reader. Finally for the *FRAP determination* the ferric reducing ability of each sample solution was  
329 estimated according to the procedure described by Benzie and Strain<sup>40</sup> and also adapted to a  
330 microplate reader.

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### 336 **Measurement of the ACEi% activity**

337 The ACE-inhibitory activity of the samples and fractions was measured following the HPLC-based  
338 method described by Gonzalez-Gonzales et al.,<sup>27</sup> with some modifications. For this aim the  
339 determination was done by RP-UHPLC, using a Thermo Scientific Accela UHPLC system (Santa  
340 Clara, USA) with thermostated compartment sample injector at 10 °C and a C18 analytical column  
341 (Extrasyl-ODS2, 250 x 4.0 mm, 5 mm, Tecknokroma, Barcelona, Spain) thermostated at 37 °C.  
342 The injection volume was 10 µL and the photodiode array detector was set at 228 nm. The flow

343 rate was 1 mL/min with an isocratic solution of acetonitrile 12.5% and trifluoroacetic acid 0.1%  
344 in milli-Q water over 8 min, as it was previously reported.<sup>41</sup>

345

### 346 **Evaluation of the antimicrobial activity**

347 This activity was studied using two bacterial strains: a Gram-negative, *Escherichia coli* K-12 (*E.*  
348 *coli*), and a Gram-positive, *Micrococcus luteus* (*M. luteus*). Before the assay all samples were  
349 filtered through 0.22 µm size pore filters (Millex® - GS, Merck Millipore Ltd., Cork, Ireland)  
350 under laminar flow and stored in sterile containers. Every measurement was done in triplicate and  
351 sterile Phosphate Buffered Saline (PBS, Sigma-Aldrich, Steinheim, Germany) was assayed as  
352 blank.

353 The antimicrobial activity of the whey, P and R fractions of StFM and St+LPFM was assayed  
354 by the well diffusion assay, based on the method described by Leon Ruiz et al.<sup>9</sup> The antimicrobial  
355 activity was also evaluated by the spot assay of antibiosis, which was carried out according to the  
356 method described by Mohankumar and Murugalatha<sup>42</sup> slightly modified. The agar was inoculated  
357 with the bacteria prepared as described above. Instead of doing wells, three 20 µL drops of each  
358 sample were put on the agar and the plates were incubated as described above. Inhibition zones  
359 were measured from the edge of the drop.

360 Finally, for the determination of the antimicrobial activity by the co-culture assay, 4.5 mL of  
361 broth culture (NB for *E. coli* and TSB for *M. luteus*), 0.5 mL of the sample and 50 µL of the  
362 bacteria suspension (growth in NB or TSB at  $\sim 6-8 \times 10^8$  cfu mL<sup>-1</sup>), were cultured all together. This  
363 mixture was incubated under stirring at 37 °C for *E. coli* and 30 °C for *M. luteus*. Aliquots at t= 0,  
364 2, 4, 8 and 24 h were taken, plated out and incubated 24h at 37°C in NA for *E.coli* and 48-72 h at  
365 30 °C in TSA for *M. luteus*. Finally, the colonies were counted and the mean for each plate was  
366 calculated and expressed as cfu mL<sup>-1</sup>.

367

## 368 **Statistical analysis**

369 The homogeneity of variances was first assessed using the Levene's test at a significance level of  
370 5% ( $p < 0.05$ ). The data normal distribution was assayed with the Shapiro-Wilk test at a  
371 significance level of 5% ( $p < 0.05$ ). Statistical analysis of data corresponding to different fractions  
372 of the same milk type was tested using the ANOVA test when the parametric conditions were  
373 fulfilled or using the Kruskal-Wallis test for non-parametric ones. Additionally, to check the  
374 existence of statistical differences between same fractions (and whey samples) from different  
375 fermented milks (with and without the probiotic) the pair wise independent t-test was used. The  
376 evaluation of the relationship between different assays was carried out by computing the relevant  
377 correlation coefficient at the  $p < 0.05$  confidence level by Pearson linear correlation (for normal  
378 distribution of data) or Spearman linear correlation (for non-normal distribution of data). Analyses  
379 were performed using SPSS 17.0 program (Windows version; SPSS Inc., Chicago, IL). The  
380 significance value  $p < 0.05$  showed the existence of significant differences.

381

## 382 **Conclusions**

383 A remarkable TAC and high ACEi activity for both fermented goat milks (StFM and St+LPFM)  
384 were found. The whey was in general one of the most active fractions in all the assays.

385 However the fractionation of the whey according to size and charge gave a very good insight into  
386 the relationship between these physicochemical properties (hence chemical structure) and activity  
387 measured as antioxidant, antimicrobial and ACEi activity. Interestingly the highest TAC measured  
388 by ORAC was found in the P<3 fraction, therefore peptides with MW<3000 Da were the main  
389 contributors to the antioxidant activity not the proteins. On the other hand, positively charged basic  
390 peptides (those in the retentate fraction of the membrane separation step) had the highest TAC  
391 against ABTS<sup>•+</sup> and DPPH<sup>•</sup> radicals; both methods test antioxidant mechanism according to HAT

392 and SET mechanisms. In terms of ACEi activity, the highest activity was found in the P<3 fraction.  
393 So the smallest (nonionic and anionic) peptides were the main contributors to the ACEi and  
394 antioxidant (according to ORAC) activities of the whey.

395 None of the samples had antimicrobial activity against the gram positive bacteria. The whey and  
396 the anionic/nonionic fractions of the fermented milk with the starter had some antimicrobial  
397 activity against the gram negative bacteria however, this may be partly due to the low pH. Only  
398 the whey and the cationic fraction of the fermented milk with the probiotic showed some activity  
399 against *E.coli* which could be attributed to peptides released by *L. plantarum* C4 during the  
400 fermentation process such as those derived from lactoferrin.

401 Finally, the activities attributed to the whey fractions show potential health benefits of the  
402 consumption of fermented goat milk. However, further research is needed to conduct clinical trials  
403 to substantiate these and for further identification of individual peptides responsible for the  
404 activities.

405

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407

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412 66886R

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**Table 1. Total protein content in the different fractions of goat fermented 470 milks (mean  $\pm$  SD, mg mL<sup>-1</sup>)**

Sample type	<i>n</i>	Whey fraction	P fraction	R fraction	P<3 KDa fraction	P>3 KDa fraction
StFM	25	6.78 $\pm$ 0.773*	5.69 $\pm$ 0.548 <sup>#</sup>	0.436 $\pm$ 0.096	2.23 $\pm$ 0.145	1.31 $\pm$ 0.377
St+LPFM	25	5.70 $\pm$ 0.661*	4.30 $\pm$ 0.843 <sup>#</sup>	0.355 $\pm$ 0.055	2.08 $\pm$ 0.127	0.97 $\pm$ 0.142
Mean value	50	6.16 $\pm$ 0.868 <sup>a,*</sup>	4.85 $\pm$ 0.990 <sup>b,#</sup>	0.388 $\pm$ 0.076 <sup>c,**</sup>	2.14 $\pm$ 0.143 <sup>d,##</sup>	1.19 $\pm$ 0.225 <sup>e,††</sup>

StFM: Fermented milk manufactured with skimmed milk concentrated by ultrafiltration (UFM) and fermented with the classical starter bacteria (St: *L. bulgaricus* and *S. thermophilus*); St+LPFM: Probiotic fermented goat milk manufactured with UFM and fermented with St and *L. plantarum* C4; Whey fraction: Fermented milk supernatant after centrifugation; P fraction: IEX (Ion exchange) permeate; R fraction: IEX retentate; P<3 fraction: P fraction with less than 3 kDa molecular weight; P>3 kDa fraction: P fraction with more than 3 kDa molecular weight.

\*.#Statistical differences between the same fractions of StFM and St+LPFM:  $p < 0.05$ .

<sup>a,b,c,d,e</sup>Superscripts with different letters indicate the existence of statistical differences among different fractions: \* $p < 0.01$ ; \*\*.#.#,†† $p < 0.001$ ).

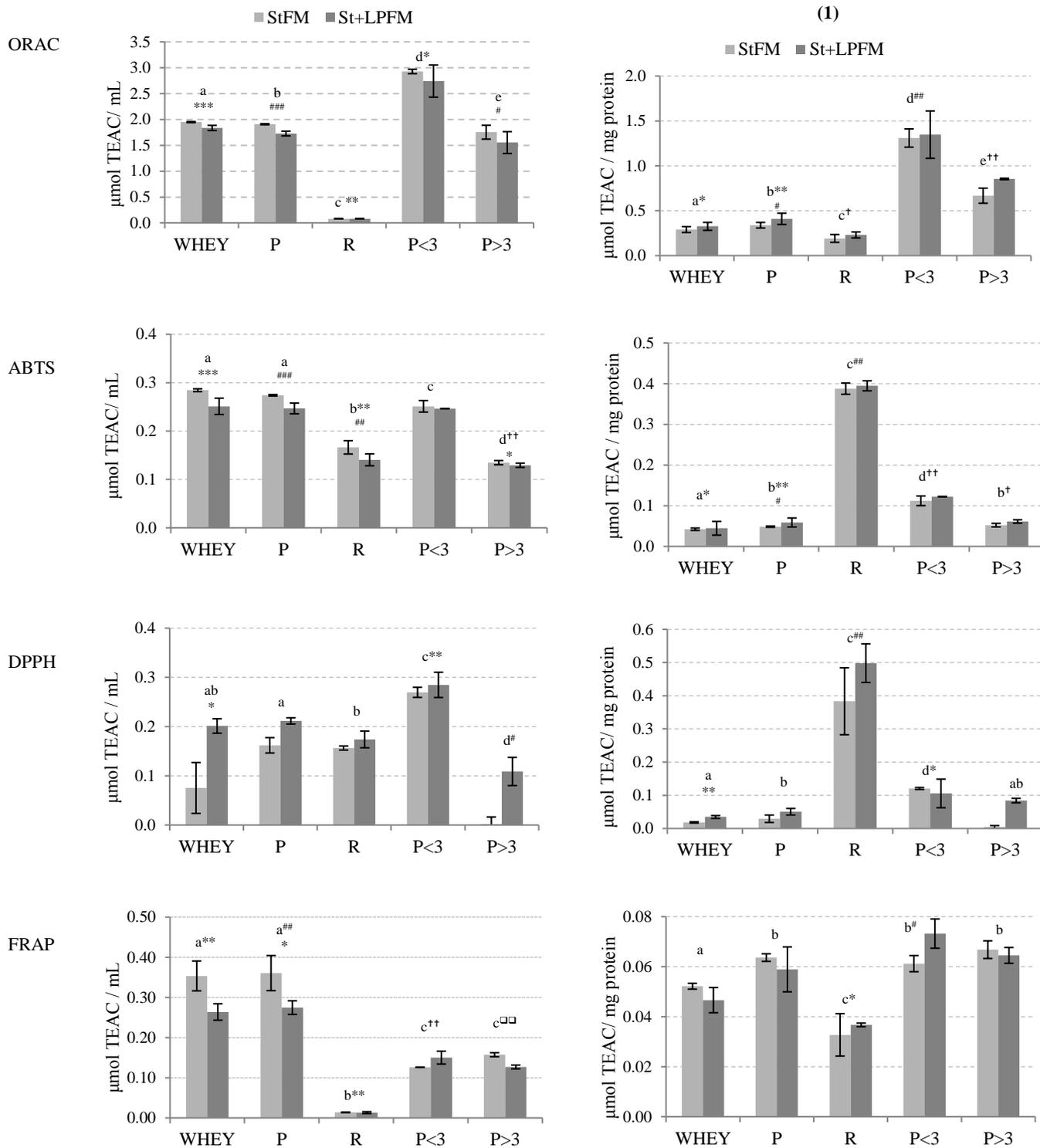
**Table 2. Final pH of the co-culture supernatants at 24h for fermented goat milks (StFM and St+LPFM) and control**

Sample	<i>n</i>	Whey fraction	P fraction	R fraction	Control
StFM (TSB)	25	5.04 ± 0.07	5.06 ± 0.01	7.46 ± 0.07	7.30 ± 0.18
St+LPFM (NB)	25	4.91 ± 0.07	4.83 ± 0.01	6.64 ± 0.01	6.85 ± 0.12

The pH was measured in the supernatant of the culture media mixed with the fractions after the assay. TSB: Tryptone soy broth culture media; NB: Nutrition broth culture media; WHEY: Fermented milk supernatant after centrifugation; P: IEX (Ion exchange) permeate; R: IEX retentate; Control: Sterile PBS.

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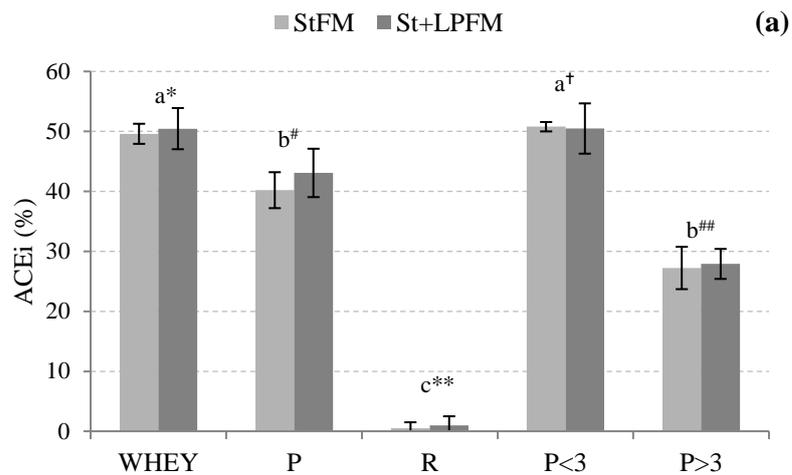
**Fig. 1. Antioxidant activity (TEAC mL<sup>-1</sup> and TEAC mg protein<sup>-1</sup>) of the fermented milk fractions by ORAC, ABTS, DPPH and FRAP assays**

StFM: Fermented goat milk manufactured with skimmed goat milk concentrated by ultrafiltration (UFM) and fermented with the classical starter bacteria (St) *L. bulgaricus* and *S. thermophilus*; St+LPFM: Probiotic fermented goat milk manufactured with UFM and fermented St and *L. plantarum* C4; Whey fraction: Fermented milk supernatant after centrifugation; P fraction: IEX (Ion exchange) permeate; R fraction: IEX retentate; P<3 fraction: P fraction with less than 3 kDa molecular weight; P>3 fraction: P fraction with more than 3 kDa molecular weight.

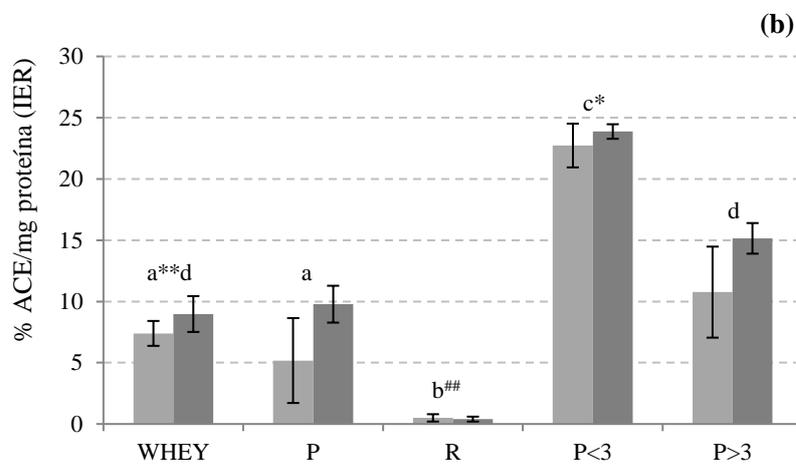
\*,†,\*\*,##,††,□□,\*\*\*,###,††† Statistical differences between values for StFM and St+LPFM: \*,†, p < 0.05; \*\*,##,††,□□ p < 0.01; \*\*\*,###,††† p < 0.001

a,b,c,d,e Superscripts with different letters indicate the existence of significant differences among fractions (letter: p < 0.05; letter,\*,#,†: p < 0.01; letter, \*\*,##,††,□□: p < 0.001).

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480 **Fig. 2. Angiotensin-I-converting-enzyme inhibitory activity (ACEi) of StFM and St+LPFM**  
 481 **expressed as percentage of ACE inhibition (a) and inhibitory efficiency ratio (IER; b).**

482 StFM: Fermented goat milk manufactured with skimmed goat milk concentrated by ultrafiltration (UFM) and fermented with the  
 483 classical starter bacteria (St) *L. bulgaricus* and *S. thermophilus*; St+LPFM: Probiotic fermented goat milk manufactured with  
 484 UFM and fermented St and *L. plantarum* C4; Whey fraction: Fermented milk supernatant after centrifugation; P fraction: IEX  
 485 (Ion exchange) permeate; R fraction: IEX retentate; P<3 fraction: P fraction with less than 3 kDa molecular weight; P>3 fraction:  
 486 P fraction with more than 3 kDa molecular weight.

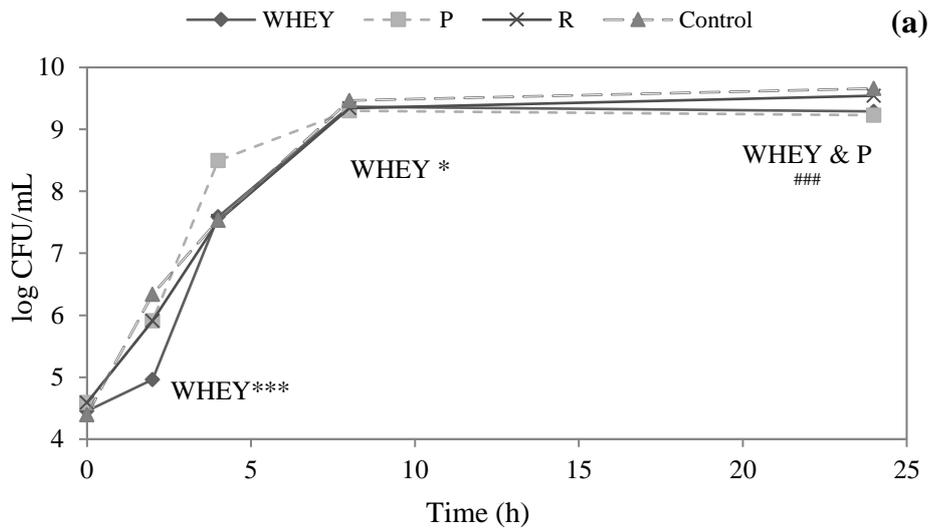
487 \*.,†,\*\*,### Statistical differences between values for StFM and St+LPFM: \*.,†  $p < 0.05$ ; \*\*,###  $p < 0.01$

488 a,b,c,d,e Superscripts with different letters indicate the existence of significant differences among fractions (letter:  $p < 0.05$ ; letter, \*,†,  
 489  $p < 0.01$ ; letter, \*\*,###,  $p < 0.001$ ).

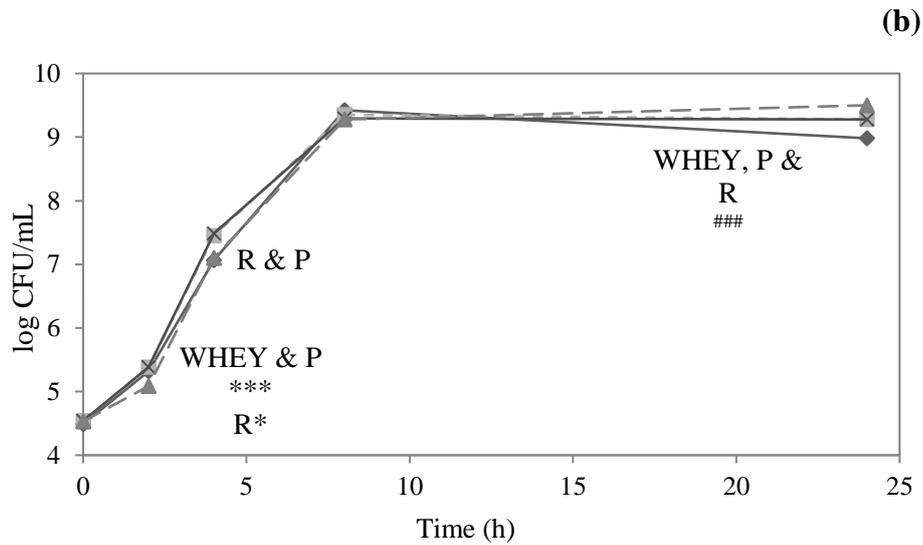
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495 **Fig. 3. Antimicrobial activity measured as viable *E. coli* after co-culture with the different**  
 496 **fractions from StFM (a) and St+LPFM (b)**

497 StFM: Fermented goat milk manufactured with skimmed goat milk concentrated by ultrafiltration (UFM) and fermented with the  
 498 classical starter bacteria (St) *L. bulgaricus* and *S. thermophilus*; St+LPFM: Probiotic fermented goat milk manufactured with UFM  
 499 and fermented St and *L. plantarum* C4; Whey fraction: Fermented milk supernatant after centrifugation; P fraction: IEX (Ion  
 500 exchange) permeate; R fraction: IEX retentate; P<3 fraction: P fraction with less than 3 kDa molecular weight; P>3 fraction: P  
 501 fraction with more than 3 kDa molecular weight; Control: sterile PBS.

502 \*\*\*\*.### Significant differences for viable *E. coli* at specific time among fractions of fermented goat milks and the control: \* $p <$   
 503 0.05; \*\*\*\*.###  $p <$  0.001.

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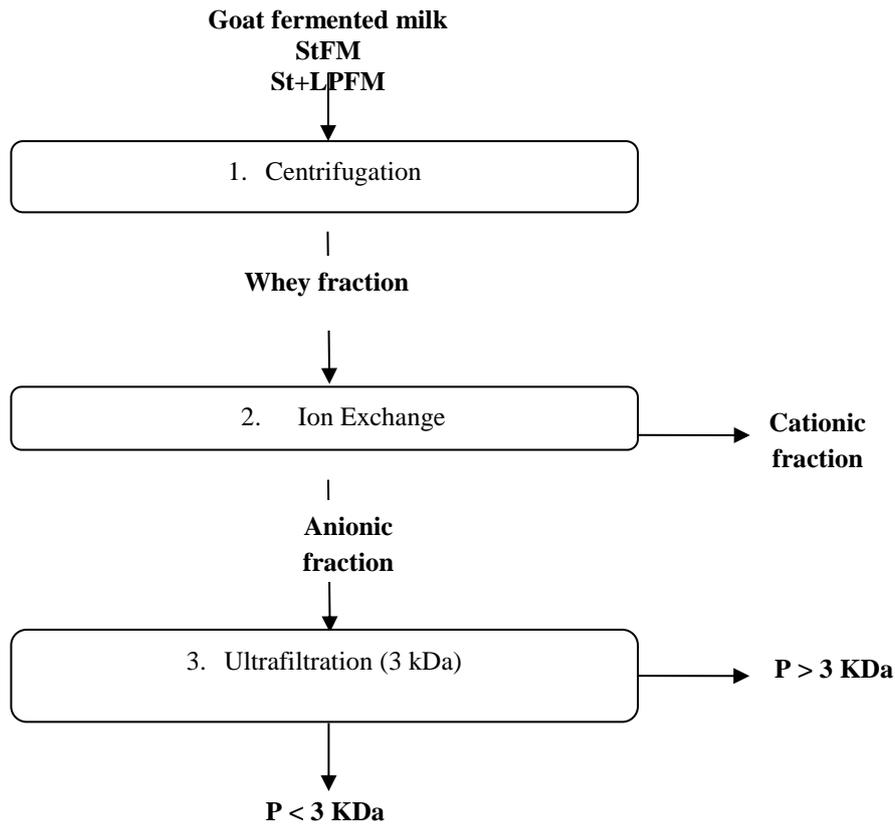
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537 **Fig. 4. Sample fractionation diagram for skimmed goat milks with classical starter bacteria**  
538 **(StFM) and with the classical starter St plus *Lactobacillus plantarum* C4 probiotic strain**  
539 **(St+LPFM)**

540 Whey: Fermented milk supernatant after centrifugation; Cationic fraction: Ion exchange (IEX) permeate; Anionic fraction: IEX retentate; P<3  
541 fraction: P fraction with less than 3kDa molecular weight; P>3 fraction: P fraction with more than 3kDa molecular weight.  
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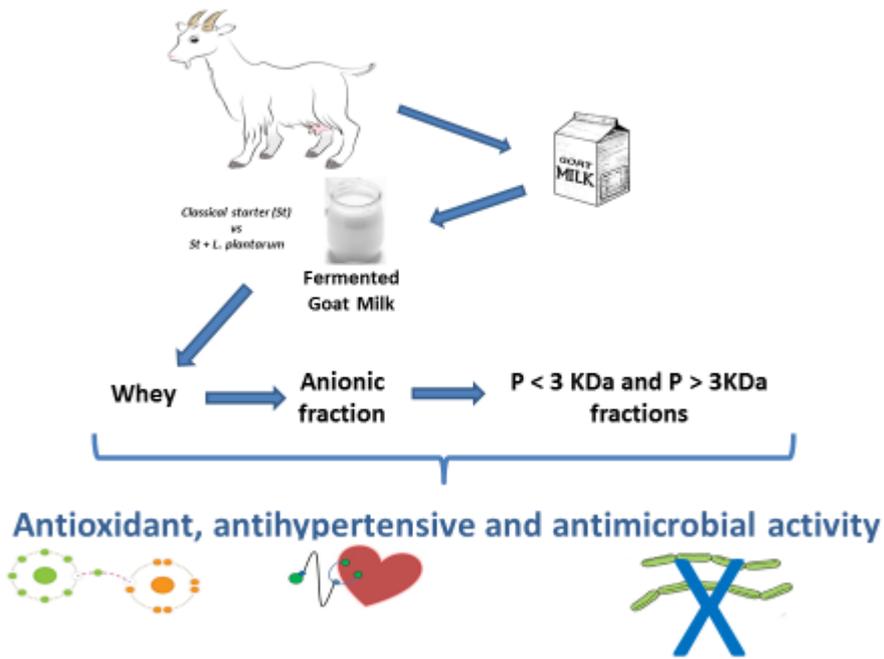
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### Graphical abstract



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