Short title: SYNERGY BETWEEN DAIRY PEPTIDES AND PROTEINS

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Summary: α_{s2}-casein f(183-207) and lactoferricin [lactoferrin f(17-41)] are two potent antibacterial peptides derived from bovine milk proteins. The aim of this work was to evaluate if the antimicrobial activity of natural compounds employed in food preservation such as nisin could be enhanced by combination with the aforementioned milk peptides. Combinations with lactoferrin were also performed. Some of the combinations used, such as lactoferrin with lactoferricin-B or lactoferrin with α_{s2}-casein f(183-207) may be relevant for the host defense properties of lactoferrin. This work further highlights the potential of using nisin in combination with α_{s2}-casein f(183-207) to improve its effectiveness at inhibiting food-borne pathogens.

Running Head: SYNERGY BETWEEN DAIRY PEPTIDES AND PROTEINS

Synergistic Effect between Different Milk-Derived Peptides and Proteins

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ABSTRACT

Antimicrobial peptides derived from food proteins constitute a new field in the combined use of antimicrobial agents in food. The best examples of milk-derived peptides are those constituted by bovine lactoferricin [lactoferrin f(17-41)] (**LFcin-B**) and bovine α_{s2}-casein f(183-207). The aim of this work was to study if the antimicrobial activity of a natural compound employed in food preservation, nisin, could be enhanced by combination with the afore-mentioned milk-derived peptides. Furthermore, the possibility of a synergistic effect between these peptides and bovine lactoferrin (**LF**) against *Escherichia coli* and *Staphylococcus epidermidis* was also studied. Finally, the most active combinations were assayed against the food-borne pathogens *Listeria monocytogenes* and *Salmonella choleraesuis*. Results showed a synergistic effect when LFcin-B was combined with bovine LF against *E. coli*. In the same way, the combination of LFcin-B with bovine LF was synergistic against *St. epidermidis.* Bovine LF and nisin increased their antimicrobial activity when they were assayed together with bovine α_{s2}-casein f(183-207). It is important to note the synergistic effect among LFcin-B and bovine LF, as both compounds might be simultaneously in the suckling gastrointestinal tract, and could, therefore, have a protective effect on it. The other synergistic effect highlighted is that between α_{s2}-casein f(183-207) and nisin against *L. monocytogenes* because of the ability of *L monocytogenes* to develop resistance to nisin.

Keywords*:* Synergism; milk-derived antibacterial peptides, antibacterial milk proteins

INTRODUCTION

Food preservation procedures such as pasteurization, refrigeration, canning, modified atmosphere packaging or the incorporation of chemical preservatives in food are usually employed to prevent the growth of bacteria that may cause human disease or food spoilage. Chemical preservatives such as benzoates, sorbates, nitrites and sulphites have been used effectively, but their safety is continually under study (Knekt et al., 1999; McCann et al., 2007). The consumer demand for minimally processed foods has led to the search for biopreservatives that can be safely incorporated into various food products. Although numerous studies have shown the effectiveness of biopreservatives against microorganisms (Altieri et al., 2005; Schnurer and Magnusson, 2005), some of them have a limited spectrum of activity, high application cost, or negative impact on the organoleptic quality of foods (Dufour et al., 2003). These limitations can, to an extent, be overcome by combinations of different antimicrobial agents (Zapico et al., 1998; Branen and Davidson, 2004), combinations of antimicrobials with chelating agents (Stevens et al., 1991), or by the use of antimicrobials together with preservative treatments such as high hydrostatic pressure, low pH or freeze/thaw cycles (Roberts and Hoover, 1996; García-Graelis et al., 2000; Cressy et al., 2003).

Nisin is a bacteriocin produced by *Lactococcus lactis* spp. *lactis* that is primarily active against Gram-positive bacteria, and it has found practical application as a food preservative in a number of food products (Delves-Boughton et al., 1996). The practical application of nisin, however, is limited because its low stability, reduced activity at high pH and poor efficacy in certain food matrices (Pol et al., 2000).

LF is a key element of the innate host defense system and, as such, it has crucial antimicrobial activities against a broad range of pathogens. In the case of bacteria, LF affects many Gram-positive and Gram-negative pathogens (reviewed in Valenti and

Antonini, 2005). In contrast, it seems to promote the growth of beneficial bacteria like Lactobacillus and Bifidobacteria (Sherman et al., 2004). The large-scale preparation of LF from cheese whey or skim milk makes it available for human and animal health purposes and commercial applications. LF also offers applications is food preservation and safety by limiting the growth of microbes. For example, incorporation of bovine LF into edible films has a great potential to enhance the safety of foods, or it can be also directly used as a spray applied to beef carcases (Taylor et al., 2004).

Antimicrobial peptides derived from food proteins constitute a new field in the use of antimicrobial agents in food. Some of them have shown potent antimicrobial activity and a broad spectrum against Gram-positive and Gram-negative microorganisms. Antimicrobial peptides have been isolated from various food proteins but the greatest number described to date are from milk (for a recent review see López-Expósito and Recio, 2006) or from chicken egg white (Pellegrini et al., 2004; Ibrahim et al., 2000). One of the most potent milk-derived antimicrobial peptides described so far corresponds to a fragment of the whey protein LF, named lactoferricin (Bellamy et al., 1992), which possesses an antimicrobial potency against a wide range of microorganisms, which is ten-fold greater than that of the parent protein. Another peptide with a strong antimicrobial activity against Gram-positive and Gram-negative microorganisms is that corresponding to the bovine α_{s2}-casein f(183-207). This fragment was obtained by hydrolysis of the bovine α_{s2}-casein with pepsin (Recio and Visser, 1999b). Although only few works deal with the synergistic effect of LF with other antimicrobial compounds such as monolaurin, lysozyme or ethylenediaminetetraacetic acid (Branen and Davidson, 2004; Ellison and Giehl, 1991), to our knowledge, no synergism has been described among milk-derived peptides and nisin and LF.

97 The aim of this work was to study whether the peptides α_{s2}-casein f(183-207)
98 and LFcin-B can exert a synergistic effect in combination with other food proteins and
99 peptides towards selected food-borne pathogens and spoilage bacteria. We intended to
100 evaluate if these two antibacterial peptides were able to destabilize the outer membrane
101 of Gram-negative microorganisms, in order to facilitate access of antimicrobial agents
102 with a limited spectrum of activity against Gram-negative microorganisms such as LF
103 and nisin.

MATERIAL AND METHODS

Bacterial Strains and Growth Media

Escherichia coli ATCC 25922 was from the American Type Culture Collection (**ATCC**) (Rockville, MD, USA), *Listeria monocytogenes* CECT 934, *Staphylococcus epidermidis* CECT 231, and *Salmonella choleraesuis ssp. choleraesuis* CECT 4594 were from The Spanish Type Culture Collection (Colección Española de Cultivos Tipo, CECT; Valencia, Spain). Tryptic Soy Broth (**TSB**), Tryptic Soy Agar (**TSA**), Brain Heart Infusion Agar (**BHIA**) and Brain Heart Infusion (**BHI**) were from Scharlau, (Barcelona, Spain). Unless otherwise stated, all other chemicals were of the highest grade commercially available.

Chemicals

Bovine LF was kindly donated by Domo Food Ingredients (Beilen, The Netherlands). Iron content of the LF preparation was determined by inductively coupled plasma-optical emission spectrometry (Larrea et al., 1997). Nisin (2.5% nisin) was purchased from Sigma (St. Louis, MO; USA).

α_{s2}-casein f(183-207) and Bovine Lactoferricin Preparation

Bovine α_{s2}-casein f(183-207) was prepared by conventional Fmoc (Fluorenyl-methoxy-carbonyl) solid-phase synthesis method with a 431 A peptide synthesiser (Applied Biosystems Inc., Überlingen, Germany) and purified after synthesis by semi-preparative RP-HPLC with the conditions previously described by López-Expósito et al (2006a).

LFcin-B was prepared as previously described by Recio and Visser (1999a). Briefly, an LF hydrolysate (5% w/v) was prepared in acidified water (pH 3.0) with 3% (w/w) of porcine pepsin A (EC 3.4.23.1, 445 U/mg solid, from Sigma) for 4 h at 37ºC. The reaction was terminated by heating at 80ºC for 15 min and the pH was adjusted to 7.0 by the addition of 1M NaOH. The supernatant obtained after centrifugation (16 000 *g* for 15 min) was injected onto a column (150 ×26 mm I.D.) of SP-Sepharose Fast Flow resin (Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated at 4ºC with ammonium hydrogen carbonate buffer acidified with formic acid to pH 7.0. Peptides were eluted with a flow rate of 5 ml/min with a gradient going from 0 to 100% in 70 min of 5 M ammonia solution. Finally, the column was eluted with 2 M NaCl, and this fraction containing LFcin-B was desalted by a semi-preparative RP-HPLC step. The purity of the LFcin-B obtained was evaluated by RP-HPLC-MS as previously described (López-Expósito et al., 2006b).

Antimicrobial Activity

Antimicrobial activity was determined using CryoTubes™ Vials (Nunc™, Roskilde, Denmark). Single colonies of bacteria grown on TSA plates (*E. coli, S. choleraesuis* and *St. epidermidis*) or BHIA plates (*L. monocytogenes*) were inoculated with 10 mL of TSB or BHI and grown overnight at 37ºC. A total of 300 µL of bacterial suspension was diluted 1/50 with TSB or BHI. Bacteria were grown at 37ºC and logarithmic phase organisms were harvested at a density of 1-4 × 10^8 colony forming units (**cfu**)/mL. The culture was then centrifuged at 2000 × *g* for 10 min. Bacteria were washed twice with 10 mM Na-phosphate buffer, pH 7.4, and adjusted to 10^5 cfu/mL approximately. A total of 50 µL of the bacterial suspension were mixed with 50 µL of the antimicrobial sample to be investigated together with 100 µL of 2% TSB or BHI in

10 mM phosphate buffer pH 7.4 and with 800 μL of 10 mM phosphate buffer pH 7.4. The mixture was incubated at 37ºC for 2 h with agitation and then plated on TSA or BHIA plates. The plates were incubated at 37ºC (*E. coli*, *S. cholerasuis*, *St. epidermidis*) or 30ºC (*L. monocytogenes*) for 24 h before the colonies were counted. The assays were conducted in triplicate. The antimicrobial activity was expressed as the concentration of antimicrobial agent that gave a log (N_0/Nf) value between 0.25 and 0.5.

Evaluation of Synergy

To determine antimicrobial interactions, a synergy index was defined based on fractional inhibitory concentration-index previously described by Davidson and Parish (1989). The synergy index of an individual antimicrobial compound is the ratio of the concentration of the antimicrobial compound in an inhibitory combination with a second compound to the concentration of the antimicrobial by itself as follows:

$$Index_A = (Activity\ of\ A\ with\ B)/Activity\ of\ A$$

The synergy index was calculated as follows with the indices for the individual antimicrobials: Synergy Index = $Index_A$ + $Index_B$. If the synergy index is <1, the interaction is considered to be synergistic, if the synergy index = 1 the interaction is additive, and an synergy index >1 represents antagonism between two substances.

RESULTS AND DISCUSSION

Determination of the antibacterial activity

The antibacterial activity of the protein and peptides under investigation was determined against two Gram-negative and two Gram-positive bacterial strains, and the results are shown in Table 1. The lantibiotic nisin was active against both Gram-

178 negative and Gram-positive bacteria although against the Gram-negative
179 microorganisms it showed notably lower activity than against the Gram-positive ones.
180 Nisin is an antimicrobial compound with a spectrum limited essentially to Gram-
181 positive microorganisms. Our results showed that nisin could also exert certain
182 antimicrobial activity against Gram-negative bacteria, confirming the results reported by
183 Kuwano et al. (2005). The disparities found by different authors are probably due to the
184 conditions of the antibacterial assay. In any case, the results reported in Table 1 show
185 that to reveal some appreciable antibacterial activity against Gram-negative bacteria,
186 nisin must be assayed at least at a concentration 10 times higher than against Gram-
187 positive bacteria.

188 LF was active against both Gram-negative and Gram-positive bacteria. The LF
189 preparation used in this study contained 198 ± 5 μg of iron/g of dry weight as
190 determined by elemental analysis (i.e., approximately 13.6% saturation, considering 2
191 metal binding sites per 77 000 Da) The antibacterial activity values ranged from 0.075
192 μM against *E. coli* to 2.5 μM against *St. epidermidis*. In fact, regarding Gram-negative
193 bacteria, LF was strongly active against *E. coli* and to a lesser extent against *S.*
194 *choleraesuis*. Among Gram-positive bacteria, *L. monocytogenes* was highly sensitive to
195 LF, whereas *St. epidermidis* was only weakly affected by the action of this protein.
196 Thus, the differences observed cannot be easily explained in terms of a different
197 composition of the bacterial membrane. These results are in agreement with previous
198 studies with human LF where non-enteropathogenic strains of *E. coli* were classified as
199 LF-sensitive strains and *St. epidermidis* was relative resistant to the effect of apo-LF
200 (Arnold et al., 1980). The peptide fragment of LF, LFcin-B, was active against both
201 Gram-negative and Gram-positive bacteria. Its antibacterial activity was stronger than
202 that of its parent protein confirming the results reported by other authors (Bellamy et al.,

1992). As shown in Table 1, similar activity was previously found for Lfcin-B against *E. coli* and *St. epidermidis* (Jones et al.,1994).

The peptide f(183-207) derived from α_{s2}-casein was active against both Gram-negative and Gram-positive bacteria. However, similarly to LF, notable differences were observed in the bactericidal activity against the strains investigated. *L. monocytogenes* was the strain most sensitive to the action of f(183-207) whereas *St. epidermidis* was the least.

Interactions Between LFcin-B and other Antimicrobial Compounds

In order to investigate a possible synergistic effect between LFcin-B and LF or nisin against *E. coli* and *St. epidermidis*, synergy indices were calculated. Results are shown in Table 2. From the results obtained, it must be highlighted that LF and the LF-derived peptide, LFcin-B, acted synergistically against *E. coli* and *St. epidermidis*. LFcin-B and LF displayed against *E. coli* activity values of 0.0125 and 0.075 μM, respectively, whereas the activity value decreased to 0.0075 μM when they were assayed together. The synergy index determined for the combination LF and LFcin-B against *E. coli* and *St. epidermidis* was 0.68 and 0.51, respectively (Table 2). The LF and LFcin-B used in this study were of bovine milk but if this synergism could also be demonstrated with LFcin and LF from human origin, it could have physiological implications. It has been demonstrated by mass spectrometry that significant amounts of fragments that contain LFcin-B are produced in human stomach following ingestion of LF, and therefore, functional quantities of human LFcin might be generated in the human stomach (Kuwata et al., 1998a). LFcin has also been detected in the gastrointestinal tract of adult mice (Kuwata et al., 1998b). In the same way, it was demonstrated that a portion of ingested LF is incompletely hydrolyzed (Spik et al.,

228 1982) and the concentration of LF in human milk is approximately 2 g/L in mature milk
229 (Lönnerdal, 2003) and 7 g/L in human colostrum (Ward and Connelly, 2004). It is,
230 therefore, likely that LF and LFcin coexist in the gastrointestinal tract of the breast-fed
231 infants and these compounds could act synergistically, increasing the host´s defences
232 against invading microorganisms.

233 When LFcin-B was combined with nisin, an antagonistic effect was found
234 against *E.coli* (FIC-index of 4.02), while the synergy index achieved against *St.*
235 *epidermidis* revealed an additive interaction (synergy index of 1.0). This antagonistic
236 effect was also previously reported when nisin was combined with reuterin against
237 Gram-negative microorganisms (Arqués et al., 2004a).

238

239 ***Interactions Between Bovine α_{s2}-casein f(183-207) and other Antimicrobial***
240 ***Compounds***

241 As shown in Table 2, the synergy indices obtained with the α_{s2}-casein peptide
242 combined with LF and nisin revealed a synergistic effect against *St. epidermidis*
243 Particularly efficient were the combinations of the α_{s2}-casein peptide with LF and nisin
244 against *St. epidermidis*, with synergy values of 0.02 and 0.1, respectively. These low
245 indices indicate a strong synergism of these two combinations. As can be observed from
246 Figure 1, when both substances were tested alone, concentrations of 10 μM of LF and 5
247 μM of the α_{s2}-casein peptide were required to reach the maximum growth inhibition.
248 When the combination of LF and the α_{s2}-casein peptide was assayed, a concentration of
249 2.5 μM of each compound was enough to obtain the same effect. If the α_{s2}-casein
250 peptide could be generated upon enzymatic hydrolysis in the suckling gastrointestinal
251 tract, this synergism might also have a physiological meaning as both compounds could
252 coexist in the gastrointestinal tract of a breast fed infant. On the other hand, the synergy

253 between α_{s2}-casein f(183-207) and nisin could find some application in the food
254 industry where nisin is already used as a food preservative. Other authors have obtained
255 a synergistic interaction by combining nisin with monolaurin (Mansour and Millière,
256 2001), garlic extract (Singh et al., 2001), lactoperoxidase system (Zapico, et al., 1998)
257 or reuterin (Arqués et al., 2004b), but to date, the interaction of nisin with other milk-
258 proteins and peptides has not been attempted. Against *E.coli*, only the combination of
259 the casein-derived peptide with LF demonstrated a synergistic interaction, while
260 combination with nisin had an antagonistic effect. It had been previously reported that
261 LF in combination with monolaurin inhibited growth of *E. coli* O157:H7 but not *E. col*i
262 O104:H21 (Branen and Davidson, 2004) and therefore, this synergistic behavior should
263 be confirmed with other *E. coli* strains.
264
265 ***Bovine α_{s2}-Casein f(183-207) Interactions Against Food-Borne Pathogens***
266 Combinations with synergy indices lower than 0.5 were also assayed against the
267 food-borne pathogens *Salmonella choleraesuis* and *Listeria monocytogenes*. The results
268 obtained for the combinations of α_{s2}-casein f(183-207) with LF and nisin are shown in
269 Table 2. These two combinations were synergistic against *L. monocytogenes* but had an
270 antagonistic effect when tested against *E. coli*. Of special interest was the combination
271 of the peptide from α_{s2}-casein with nisin, because of the ability of *L. monocytogenes* to
272 develop resistance to nisin (Davies and Adams, 1994). Probably, the peptide α_{s2}-casein
273 f(183-207) could destabilize the bacterial membrane, making this microorganism more
274 susceptible to the action of nisin. Therefore, as indicated above, the combination of α_{s2}-
275 casein f(183-207) and nisin could be of use in the food industry as a food preservative.
276 In relation to *S. choleraesuis*, none of the combinations assayed were synergistic
277 against this bacterium. The synergy index was 1.75 for the combination with LF and

278 5.50 for the combination with nisin (Table 3). The reason why these two combinations,
279 casein-derived peptide with LF or nisin, were synergistic against the Gram-positive
280 bacteria (*St. epidermidis* and *L. monocytogenes*) but not against Gram-negative bacteria
281 (*E. coli* and *S. choleraesuis*) is not clear. It may be due to the more complex membrane
282 structure of Gram-negative bacteria. However, combinations of LF with LFcin-B or
283 with the casein-derived peptide exerted a synergistic effect against *E. coli*. It has been
284 postulated that differences in the antibacterial action of EDTA-nisin combinations
285 against different Gram-negative bacteria could be attributed to differences in the outer
286 membrane or LPS structure which may affect the amount of LPS released from the
287 outer membrane and the resulting increase in permeability (Branen and Davidson,
288 2004).
289
290 **CONCLUSIONS**
291 The antimicrobial activity of LF and nisin can be enhanced by simultaneous
292 addition of the peptides LFcin-B and α_{s2}-casein f(183-207). More specifically, these
293 two peptides have been demonstrated to act synergistically or additively with LF and
294 nisin against the Gram-positive microorganism *St. epidermidis*. However, against
295 Gram-negative *E. coli*, only the combination of these two peptides with LF have proved
296 to be more effective at inhibiting bacterial growth than either agent used alone. Peptide
297 α_{s2}-casein f(183-207) synergistically enhanced the activity of nisin and LF against *L.*
298 *monocytogenes*. Some of these combinations, such as LF with LFcin-B or LF with α_{s2}-
299 casein f(183-207) may be relevant for the host defense properties of LF. The results
300 obtained in this work further highlight the potential of using nisin in combination with
301 α_{s2}-casein f(183-207) to improve its effectiveness at inhibiting *Listeria monocytogenes*.

Although results obtained in growth media cannot be directly extrapolated to food matrices, this combination may, therefore, be promising for use in food preservation.

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412

413 Figure captions

414 **Figure 1.** Antibacterial activity at different concentrations of (■) lactoferrin (◆) α_{s2}-
415 casein f(183-207) (▲) lactoferrin + α_{s2}-casein f(183-207) against *Staphylococcus*
416 *epidermidis* CECT 231 growth in tryptic soy broth. Antibacterial activity was calculated
417 as log N_0/N_f. Where N_0 refers to the control number of colonies without antibacterial
418 material (10^3 cfu/mL) and N_f refers to the number of colonies containing antibacterial
419 compounds after an incubation period of 2 h at 37ºC.

Figure 1

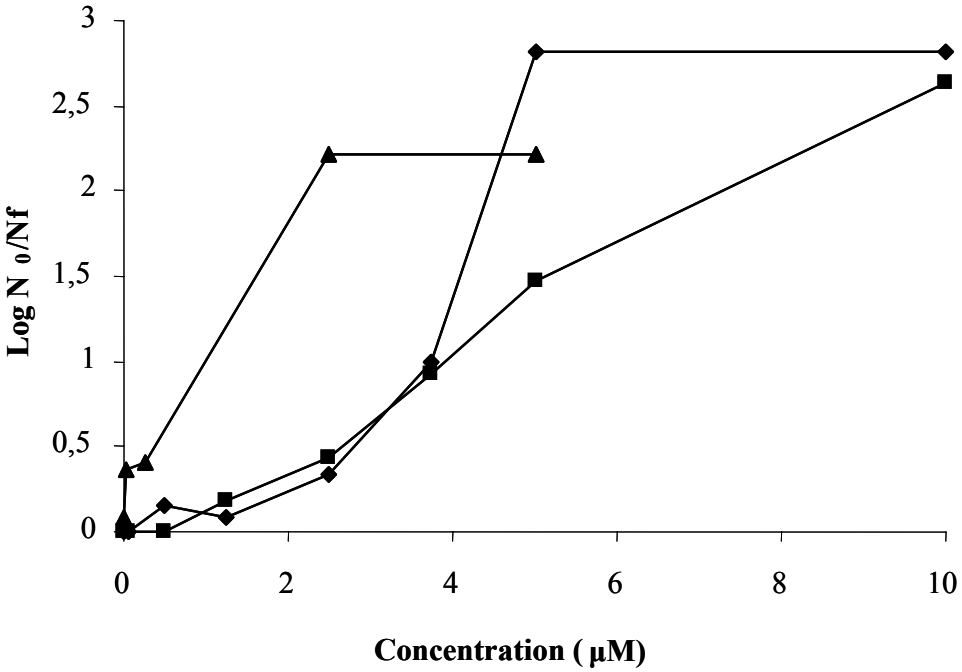

Table 1. Antibacterial activity expressed as the concentration (μM) of antimicrobial agent that gave a log (N_0/Nf) value between 0.25 and 0.5 against different Gram-negative (*Escherichia coli, Salmonella choleraesuis*) and Gram-positive (*Staphylococcus epidermidis, Listeria monocytogenes*).microorganisms for each antimicrobial agent evaluated.

Antimicrobial	Microorganisms			
	Gram-negative		Gram-positive	
	E. coli	*S. choleraesuis*	*St. epidermidis*	*L. monocytogenes*
LF	0.075	1.25	2.500	0.25
Nisin	0.500	5.00	0.050	0.25
f(183-207)	1.250	0.5	2.500	0.05
LFcin-B	0.0125	n.d	0.050	n.d

n.d: not determined

Molecular masses considered were 77000 for lactoferrin, 3475 for nisin; 3115 for α_{s2}-casein f(183-207), and 3125 for bovine lactoferricin.

447 **Table 2.** Antibacterial activity expressed as the concentration (μM) of antimicrobial
448 agent that gave a log (N_0/Nf) value between 0.25 and 0.5, and synergy indexes (index)
449 for each combination assayed against *Escherichia coli* ATCC 25922 and
450 *Staphylococcus epidermidis.* Combinations of α_{s2}-casein f(183-207) with lactoferrin (LF)
451 and nisin were also assayed against *Salmonella choleraesuis* and *Listeria monocytogenes*.
452

Antimicrobial	Microorganisms					
	Escherichia coli			*Staphylococcus epidermidis*		
LFcin-B with	Activity	Index	Effect	Activity	Index	Effect
LF	0.0075	0.68	Synergism	0.0025	0.51	Synergism
Nisin	0.0500	4.02	Antagonism	0.0250	1.00	Additive
α_{s2}-casein f(183-207) with	Activity	Index	Effect	Activity	Index	Effect
LF	0.025	0.35	Synergism	0.0250	0.02	Synergism
Nisin	2.500	7.00	Antagonism	0.0050	0.10	Synergism
	Salmonella choleraesuis			*Listeria monocytogenes*		
α_{s2}-casein f(183-207) with	Activity	Index	Effect	Activity	Index	Effect
LF	2.5	1.75	Antagonism	0.0025	0.60	Synergism
Nisin	0.0625	5.50	Antagonism	0.0025	0.60	Synergism