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Research Article

DETERMINING THE SYNERGISTIC CHARACTER OF ANTIMICROBIAL PEPTIDES - LACTOFERRIN AND PARASIN TESTED AGAINST MASTITIS CAUSING BACTERIA

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ABSTRACT

Antimicrobial peptides (AMPS) like Lactoferrin and parasin was selected to determine synergistic antibacterial activity against mastitis causing pathogens was framed as a primary objective in the study. The synergistic activity of a lactoferrin and a parasin on the test bacteria was determined by the standard checker board titration method. The results revealed that *Staphylococcus aureus* and *Escherichia coli* showed complete synergistic effect for lactoferrin and parasin combination. *E. coli* showed complete synergy with the mean MIC value 0.03 μg/ml and with best FICI value 0.24 ($p<0.5$). And *S. aureus* showed complete synergy with the mean MIC value 0.12 μg/ml and with best FICI value 0.72 ($p<0.5$). The synergistic antibacterial activity of AMPs in combination was reported with substantial evidence against a wide variety of Gram-negative and Gram-positive bacteria. The antibacterial activity of AMPs obtained from lactoferrin and parasin in combinations was evaluated against the test organisms by well diffusion method. Maximum inhibitory zones were recorded for lactoferrin and parasin (50μg concentration). Depending on the concentration gradient of AMPs, increase in inhibitory zones was recorded. Inhibitory zones of about 9mm, 12mm, 18mm and 22mm was obtained for its respected concentrations (20μg, 30μg, 40μg and 50μg) against *E. coli*. Similarly, respected inhibitory zones of like 9mm, 11mm, 19mm and 21mm was obtained for the tested concentrations viz., 20μg, 30μg, 40μg and 50μg) against *S. aureus.* The obtained results were substantially supported when compared with the checker board titration method to describe the synergistic characters of the tested AMPs.

Keywords: Antimicrobial peptides, Lactoferrin, Parasin, Checker board titration method, Synergism

INTRODUCTION

Antimicrobial peptides (AMPs) are the main component of biochemical defense systems[1]. Marine AMPs (mAMPs) have mainly been investigated in economically important species with the praiseworthy goal of achieving better understanding of the host's natural defense. Parasin is the mAMP extracted from the *Parasilurus asotus* (catfish). It is a potent 19-residue antimicrobial peptide isolated from the skin mucus of wounded catfish (*Parasilurus asotus*). Parasin I does not show hemolytic activity and has broad range of inhibition activity. It is about 100 times stronger than magainin. Furthermore, parasin I show good antimicrobial activity against fish-specific bacterial pathogens[2]. These peptides are potentially effective alternative therapeutants. AMP activities against viruses, bacteria, fungi and parasitic cells and may act at different stages during the course of viral infection to inhibit viral replication[3].

Lactoferrin is a group of iron-binding proteins belonging to transferrin. They show broad spectrum of antibacterial activity against Gram negative and Gram-positive bacteria. The mechanism of the antibacterial activity of lactoferrin is complex and involves beside iron-chelation, direct action on bacteria and/or the activation of the immune system. Lactoferricin and other derivatives from lactoferrin are potent antibacterial agents which penetrates the bacterial membrane[4].

Lactoferrin is a multifunctional glycoprotein present neutrophil. Lactoferrin shows strong antibacterial activity against *Streptococcus* sp., *Enterococcus* sp., *Staphylococcus* sp., *E. coli* sp., and *Haemophilus influenzae*[5]. However, lactoferrin can also promote the growth of bacterial species like *Lactobacillus and Bifidobacteria*. Mechanisms involved in antibacterial activity involves iron-chelation[6], interaction with bacteria[7], proteolytic activity[8] and stimulation of the immune response[9].

It showed that greater antibacterial effect was achieved with lower concentration of AMPs. This assumption agreed with that of the following factors described by Saginur[10] *et al.,* (2006). They described that the combination therapy would be effective in treatment of biofilm associated infections blended at different combinations. By this method broader spectrum of activity is achieved and lower concentrations of the antimicrobial are required, resulting in more effective therapy and decreased resistance to drugs[11].

Based on the approach of Gorman and Jones[11], (2002) in the present research Lactoferrin and parasin was selected to determine its synergistic antibacterial potential against bacterial pathogens causing mastitis. Lactoferrin and parasin were significantly used to treat both aerobic and anaerobic infections based on their different bactericidal actions. The rationale of combination of Lactoferrin and parasin was based on the fact that both their derivatives act synergistically. This could be due to

different bactericidal actions like iron-chelation, interaction with bacteria, proteolytic activity and stimulation of the immune response of the extracted AMPs in the present research.

MATERIALS AND METHODS

Live *Parasilurus asotus* (cat fish) was obtained from a local fish market in Coimbatore, Tamilnadu, India. Microorganisms (*Escherichia coli* and *Staphylococcus aureus*) used in this study were procured from Division of Microbiology, School of Biological Sciences, CMS College of Science and College, Tamilnadu, India. The entire work was carried out in Division of Microbiology, CMS College of Science and College, Coimbatore from January 2019 to April 2019.

Extraction of Anti-microbial peptides from Whey and *Parasilurus asotus*

Bovine Lactoferrin was extracted from cheese whey using the method described by Park[12] *et al.,* 1998. Skin of the catfishes were injured (16 cm²) with a sandpaper and after 5h, the catfishes were stunned by electro-shock. From both the unwounded and wounded catfishes, the proteinaceous epithelial mucosal layer was scraped off. 20g of mucus was collected from the skin and homogenized using a Waring blender (Waring, New Hartford, CT, USA) in 200 ml of extraction medium (0.2 M sodium acetate, 0.2% Triton X-100, and 1 mM phenyl methyl sulfonyl fluoride). The homogenate was centrifuged at 20,000Ug for 30 min (Himac SCR20BR) and the supernatant was collected and stored.

Separation of bioactive compounds using silica gel Column-chromatography method

A long cylindrical glass column (450mm X 20mm) should be stand firm on a column-chromatography stand was selected for the present research. Silica gel (60 - 120 mesh) was packed with the aid of hexane without any air bubbles. The extracts were distilled dried and finely powdered form for easy distribution of sample in already packed silica gel column. Sample powdered mass was placed on the top of the pre-packed silica column and sample was covered with a layer of cotton. Then solvents (100% hexane) were passed through column at uniform rate under gravity to fractionate the sample extract. Each fraction was collected separately in a test tube and numbered consecutively for further analysis and about 10 different fractions were collected. This protocol was presented elaborately in our previous paper[13].

Determining the Synergistic activity of AMPs against Mastitis causing bacteria[14]

The synergistic activity of a lactoferrin AMP and a parasin AMP on all the test bacteria was determined by the standard checker board titration method. To determine the inhibitory concentrations of each AMP separately and in combinations, the minimal inhibitory concentrations (MIC) was simultaneously identified in this method. The fractional inhibitory concentrations (FIC) of the AMPs were calculated from MIC values to determine the synergism between the lactoferrin AMPs and parasin AMPs.

Assessing the antimicrobial combinations against test bacteria using standard checker board titration method[15]

To assess antimicrobial combinations *in vitro* the checkerboard method was selected. In this technique by using agar dilution method, the concentrations tested for each antimicrobial agent were typically ranged from four or five below the expected MIC to twice the anticipated MIC as in the 45-degree line in Fig- 1 (each square represents one plate).
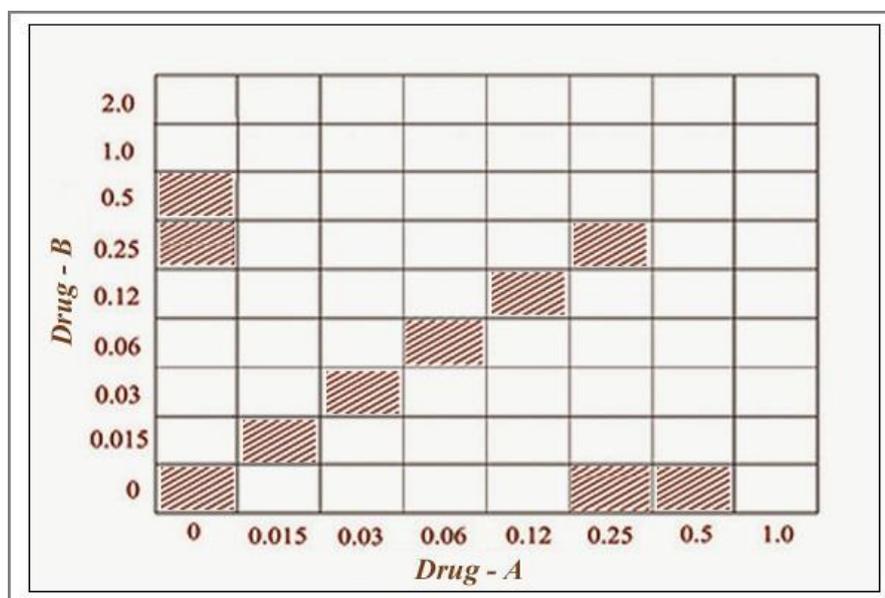

Fig-1: Checkerboard model to determine synergism of two drugs
(The picture was adapted from Qaziasgar and Kermanshahi, 2008)
Bottom row: To determine MIC of Lactoferrin (Drug-A), Left column: to determine MIC of Parasin (Drug-B),
Centre: MIC of (Drugs) AMP A + B

The predetermined concentrations (μg/ml) used for this method were 0.015, 0.03, 0.06, 0.12, 0.25, 0.5, 1.0 and 2.0. According to Fig-1, the plates in the left-hand column was used for the predetermined concentrations of first AMP (lactoferrin), the plates in bottom row was used for second AMP (parasin) and the plates in the 45-degree line was used for mixed AMP combinations. In all the arranged plates, 1ml of predetermined dilutions of the antimicrobial agents were added with sterile and molten Muller-Hinton agar. Then the surface of each plate was inoculated with 1 X 10⁴ CFU/spot of bacteria. After 16-20 hours

incubation at 37 ℃, the plates were examined for evidence of visible growth. Experimental set up was made for all the AMP combinations (lactoferrin – parasin) in triplicate.

Evaluating the synergism between lactoferrin and parasin AMPs by fractional inhibitory concentration index[16]

Fractional inhibitory concentration index (FICI) was calculated by using the following equation.
Formula to determine synergy
FIC *index* = FICA + FICB

$$FICA = \frac{MICA \text{ in combination}}{MICA}$$

$$FICB = \frac{MICB \text{ in combination}}{MICB}$$

where, A was the minimal inhibitory concentration (MIC) of AMP A in a plate that was the lowest inhibitory concentration in its row, and B was the MIC of AMP B in a plate that was the lowest inhibitory concentration in its column. MICAB was the lowest inhibitory concentration of AMP A and B in combination in the 45-degree line. With this method, synergism has traditionally been defined as an FIC index of 0.5 or less and partial synergy as a FIC index of >0.5 - ≤1.0; antagonism has been defined as a FIC index of ≥2.0.

Interpretation:
Mean FICI ≤ 0.5 → Synergy, ($p < 0.5$),
Mean FICI >0.5 - ≤1.0→ Partial synergy, ($p > 0.5$)
Mean FICI ≥ 2.0→ Antagonism

Synergy: Synergistic action of a combination of AMPs is present if the effect of the combination exceeds the additive effects of the individual components.

In simple terms, synergism is defined as the ability of a pair of AMPs to produce a more rapid rate of bactericidal action within the first 24 hours of exposure than either member of the pair alone, and killing of great numbers of bacteria that could be expected from simple summation of single AMP effects.

Partial synergy: The additive effect of combination is one in which the effect of combination is equal to that of the sum of the effects of the individual components.

Qualitative Antibacterial activity of synergistic AMPs

The antibacterial activity of AMPs obtained from lactoferrin and parasin in combinations was evaluated against the test organisms by well diffusion method. Sterile Nutrient Agar (Composition g/L: Peptone: 5g; Yeast extract: 5g, Beef extract: 3g, Sodium chloride: 5g, Agar 15 g; Final pH (7.0 ± 0.2) plates were prepared and allowed to solidify. About 0.1% inoculum suspensions of the test organism one gram positive (*Staphylococcus aureus*) and one gram negative and (*Escherichia coli*) were swabbed uniformly over the agar surface. Under sterile conditions, 6mm wells were cut on the agar surface of each NA plates. About 50μl each of each fraction were loaded into the well and the plates were incubated at 37℃ for 24 - 48h. The antibacterial activity was evaluated in terms of zone of inhibition around the wells of each extract in all the inoculated NA plates. The inhibition clear zones were measured and recorded in millimeter.

RESULTS AND DISCUSSION

Determining the synergistic activity of lactoferrin and parasin AMPs against test bacteria

Minimal inhibitory concentration (MIC) of lactoferrin (AMPA) and parasin (AMPB) was determined against all the test organisms.

Table-1 showed that the test organisms [*S. aureus* and *E. coli*] showed complete synergistic effect for lactoferrin and parasin combination. *E. coli* showed complete synergy with the mean MIC value 0.03 μg/ml (Fig-2) and with best FICI value 0.24 (p<0.5). *S. aureus* showed complete synergy with the mean MIC value 0.12 μg/ml (Fig-3) and with best FICI value 0.72 (p<0.5).

The MIC of the AMP combination obtained for all the three was very low in comparison with the individual effect of each AMP. It showed that greater antibacterial effect was achieved with lower concentration of AMPs. This assumption agreed with that of the following factors described by Saginur[10] *et al.,* (2006). They described that the combination therapy would be effective in treatment of biofilm associated infections blended at different combinations. By this method broader spectrum of activity is achieved and lower concentrations of the antimicrobial are required, resulting in more effective therapy and decreased resistance to drugs[11].

Table-1: Effect of lactoferrin + parasin against test bacteria

Test Bacteria	MICA	MICB	MICAB	FICA	FICB	FICAB	Index
Escherichia coli	0.25	0.25	0.03	0.12	0.12	0.24	S
Staphylococcus aureus	0.25	0.5	0.12	0.48	0.24	0.72	S

Mean value of three trials were tabulated
A - Lactoferrin B - Parasin, AB- Combined concentration of Lactoferrin and Parasin
S – Synergy, PS – Partial synergy
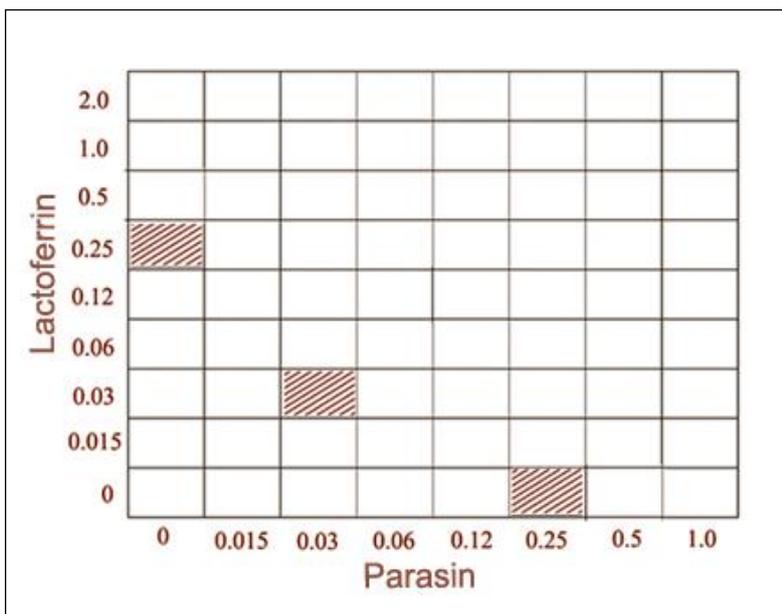Units for all the presented values - μg/ml

Fig-2: Simplified Checker Board Method of lactoferrin + parasin against *E. coli*
MICA – 0.25, MICB – 0.25, MICAB – 0.03
Test Bacteria: *E. coli*
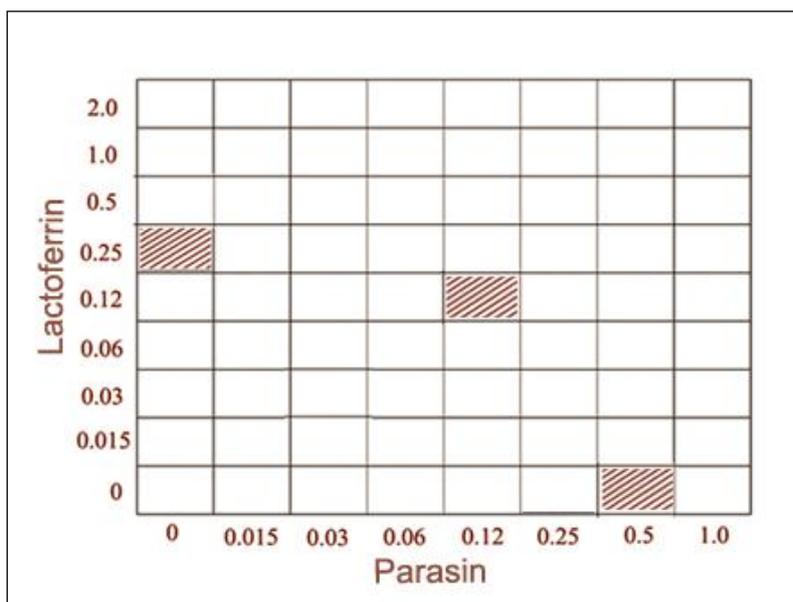Tested in triplicates

Fig-3: Simplified Checker Board Method of lactoferrin + parasin against *S. aureus*
MICA – 0.25, MICB – 0.25, MICAB – 0.03
Test Bacteria: *S. aureus*
Tested in triplicates

Lactoferrin and parasin were significantly used to treat both aerobic and anaerobic infections based on their different bactericidal actions. In the present research, the two AMP combinations proved synergistic characters against the test bacterial cultures [*S. aureus* and *E. coli*]. The rationale of combination of Lactoferrin and parasin was based on the fact that both their derivatives act synergistically. This could be due to different bacteriostatic and bactericidal actions like iron-chelation, interaction with bacteria, proteolytic activity and stimulation of the immune response of the extracted AMPs in the present research.

Synergistic bactericidal and/or bacteriostatic activity of AMPs based on the Iron-chelation factor was described in detail. The high affinity of AMPs for iron, allows the synergistic peptides to produce an iron-deficient environment which further limits the growth of target bacteria. The receptors of AMPs contain an outer-membrane protein called LbpA and a surface lipoprotein called LbpB. The interaction of LpbA induces conformational changes in the C-lobe of protein; this mechanism results in the release of iron further results in complete depletion of iron in the bacterial cells. The same concept was reported in the pathogen, *P. gingivalis.* LbpA of Lactoferrin binds to the haem receptor

protein (HbR) of *P. gingivalis* and disturbs the iron uptake behavior of organisms from hemoglobin[17].

Apart from this bacteriostatic action of AMPs, Lactoferrin and Parasin also reported to exhibit bactericidal activity which was significantly distinct from its iron-with holding capacity. The molecular mechanism was found to be similar for both Gram-negative and Gram-positive bacteria, the mechanism involves a direct interaction of AMPs with the bacterial cell membrane. Similar effect was reported by Sallman[18] *et al.* (1999); the direct interaction of Lactoferrin with the negatively charged region of OmpC and PhoE porins on *E. coli* surface leads to bacterial cell membrane damage. Pores were induced on the membrane due to this interaction leading to release of cytoplasmic constituents from the bacteria. The AMPS act by binding through electrostatic interactions to the negatively charged lipid matrix of the Gram-positive bacterial membrane leading to similar bactericidal effects occur in Gram-negative bacterial membrane.

Another significant synergistic character of Lactoferrin and parasin was displayed as serine protease activity. The cleavage of two colonizing factors, IgA$_1$ protease protein and Hap adhesion were the proteolytic activity involved. This activity of Lactoferrin observed in the N-terminal lobe of lactoferrin. The virulence factors, such as IpaA and IpaC from *Shigella flexneri* and secreted proteins A, B, D from *E. coli* were also degraded[19].

Qualitative Antibacterial activity of synergistic AMPs

The antibacterial activity of AMPs obtained from lactoferrin and parasin in combinations was evaluated against the test organisms (*Staphylococcus aureus and Escherichia coli*) by well diffusion method.

During the analysis, all the five concentration combinations exhibited inhibitory zones against the test organisms. Maximum inhibitory zones were recorded for lactoferrin and parasin (50µg concentration). Depending on the concentration gradient of AMPs, increase in inhibitory zones was recorded. Inhibitory zones of about 9mm, 12mm, 18mm and 22mm was obtained for its respected concentrations (20µg, 30µg, 40µg and 50µg) against *E. coli* (Figure-4). Similarly, inhibitory zones of like 9mm, 11mm, 19mm and 21mm was obtained for the tested concentrations viz., 20µg, 30µg, 40µg and 50µg) against *S. aureus* (Table-2). No inhibitory zones were obtained for 10µg against both *E. coli* and *S. aureus* (Figure-5). The obtained results clearly indicated the behaviour of synergism between lactoferrin and parasin. The obtained results were substantially supported when compared with the checker board titration method to describe the synergistic characters of the tested AMPs.

Table-2: Zone of inhibition of different concentration combinations of lactoferrin and parasin

S. No.	Concentration of AMPs in combination (µg)	Zone of inhibition (mm)	
		Escherichia coli	*Staphylococcus aureus*
1	10	-	-
2	20	9	9
3	30	12	11
4	40	18	19
5	50	22	21

Figure-4: Antibacterial activity of different concentration combinations of lactoferrin and Parasin against *Escherichia coli*

Figure-5: Antibacterial activity of different concentration combinations of lactoferrin and Parasin against *Staphylococcus aureus*

The synergistic antibacterial activity of AMPs in combination was reported to inhibit a wide variety of Gram negative and Gram-positive bacterial species. The activity is due to the disruption of bacterial membrane. The lipopolysaccharide (LPS) interacts with the amino acid residues 28–34 in the loop region and homologous region of AMPs of bacteria. Elass-Rochard[20] *et al* (1995) reported in their studies that Lactoferrin and synthetic peptides undergo hydrophobic interactions with tryptophan and arginine residues, which acts as the major determinants of antibacterial activity.

The mode of action proposed as the interaction of positively charged residues of lactoferrin with negative charges present in the inner core of lipopolysaccharide (LPS) of bacteria. These interactions disorganize the structure of the outer membrane and facilitate the approach of tryptophan residues to lipid A and promote hydrophobic interactions. These results were found evident against *B. subtilis, E. coli, and P. aeruginosa.* Flores-

Villasenor[21] *et al.,* 2010 described that interaction and membrane disruption are the probable mechanisms involved in the bactericidal effect on the antibiotic-resistant strains of *S. aureus* and *E. coli.*

Similar antibacterial action of parasin was reported as like Lactoferrin in many surveys. Parasin I was found only in the skin mucous extracts of the injured catfish; indicating that the skin stimulated parasin I secretion into the mucous layer as a thick gel-like layer of proteinaceous materials, which includes antibodies and lysozyme[22]. The antimicrobial peptides are stored in the granular glands and release the contents onto the epithelia upon adrenergic stimulation. Parasin I showed a strong antimicrobial activity against both Gram-negative bacteria, Gram-positive bacteria. At concentrations of 0.25-4µg/ml, most antimicrobial peptides kill the susceptible bacteria. The antimicrobial property of most antimicrobial peptides is generally attributed to their amphipathic secondary structures with a net positive charge. They act on the sensitive cells by disrupting the plasmatic membrane of the organisms[23].

Major antibacterial activity of Lactoferrin, parasin and other common AMPs were reported based on the type specific group of bacteria. The mode of action of AMPs on Gram-positive bacteria was specified as Iron-independent interaction with bacterial cell surface, interaction with lipoteichoic acid on bacterial surface and prevents biofilm formation through iron sequestering. In Gram-negative organisms the mode of actions are cation chelators, damaging the bacterial membrane, altering the outer membrane permeability which results in the release of LPS. Degrading bacterial virulence factors like IgA1, IpaB, IpaC and Hap, disrupting bacterial type III secretion system.

CONCLUSION

In the present research Lactoferrin and parasin was selected to determine its synergistic antibacterial potential against bacterial pathogens causing mastitis. The mode of action of lactoferrin in antibacterial activity is complex and several lines of evidence indicate that beside iron-chelating it involves a direct action on bacteria and the immune system is activated. Lactoferrin and Parasin were found to be more potent antibacterial agents, involved in penetration of bacterial membrane. The antibacterial activity of AMPs obtained from lactoferrin and parasin in combinations was evaluated against the test organisms (*Staphylococcus aureus and Escherichia coli*) by well diffusion method. The obtained results clearly indicated the behaviour of synergism between lactoferrin and parasin. The obtained results were substantially supported when compared with the checker board titration method to describe the synergistic characters of the tested AMPs. The rationale of combination of Lactoferrin and parasin was based on the fact that both their derivatives act synergistically. This could be due to different bacteriostatic and bactericidal actions like iron-chelation, interaction with bacteria, proteolytic activity and stimulation of the immune response.

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