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Original Article

Activity of breast milk antimicrobial peptides in experiments *in vitro*

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ABSTRACT

The most common antimicrobial polypeptides in breast milk are lactoferrin (LF), lactalbumin (LA), lysozyme (LC), and lactoperoxidase (LP). The aim of the present study was to evaluate the combined effect of these polypeptides on the cells of *Candida albicans, Staphylococcus aureus,* and *Escherichia coli in vitro* by spectrophotometric method. The antimicrobial effect of LP on the microbial cells was tested without connection with the lactoperoxidase system. It was shown that LA alone did not demonstrate any antimicrobial activity. However, the LF, LP, and LC in the concentration range from 2.5 mg/ml to 20 mg/ml exhibited a direct microbicidal effect in a significantly dose-dependent manner. The combined effect of the preparations was studied at a concentration of 5 mg/ml. The total antimicrobial activity of the combination of LF and LC on *S. aureus* and *E. coli* was significantly lower than the sum of the activities of individual preparations, i.e., there was an antagonistic effect, whereas there was a slight synergy towards *C. albicans*. For the LF and LP pairs, the experimental and calculated values of the total activity turned out to be almost the same against both types of bacteria, whereas in the case of yeast, there was a significant antagonistic effect. For the pair LP and LC in both species of bacteria, there was an antagonistic effect, and in relation to *C. albicans*, these results were practically the same. Thus, the combined actions of all the three antimicrobial peptides have shown a prominent antagonistic effect.

KEYWORDS: antimicrobial peptides, breast milk, lactoferrin, lactoperoxidase, lysozyme, and milk formula.

INTRODUCTION

Breast milk is not only a source of nutrients for the baby but also a protective factor against several pathogens [1]. Antimicrobial properties of breast milk are due to a set of cellular and humoral factors, and low molecular weight antimicrobial (poly) peptides (AMP), such as lactoferrin, lysozyme, defensins, cathelicidin, lactoperoxidase, dermcidin, hepcidin, lactalbumin, etc., that play a special role in such protection [2, 3]. It has been reported that the concentration of AMP in mother breast milk is up to 7 mg/ml for lactoferrin, up to 4 mg/ml for lactalbumin, almost 0.9 mg/ml for lysozyme, and 0.8 mg/L for lactoperoxidase [4-7]. Other AMPs are found in breast milk in nanogram quantities. Artificial milk formulas, especially for feeding premature babies, besides nutrients, can be additionally enriched with lactoferrin [8-10]. Lactoferrin, when used in healthy volunteers, has shown good tolerance even at high concentrations [11]. The antimicrobial activity of lactoperoxidase in combination with thiocyanate and hydrogen peroxide (LPO) allows its active use in the dairy industry to preserve the spoilage of raw milk and dairy

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products, even if the storage temperature is violated [12, 13]. The addition of other antimicrobial polypeptides can improve the quality of these mixtures; however, it is not known yet how these polypeptides will interact with each other when acting together against opportunistic microbiota.

Lactoferrin (LF) is an 80 kDa glycoprotein containing about 690 amino acids and two iron atoms [14]. LF is an acute-phase protein, which is synthesized in neutrophils and epithelial cells, providing antioxidant, anti-inflammatory, and antitumor effects on cells, as well as antimicrobial and antiparasitic effects on bacteria, protozoa, viruses, and fungi [15-20]. Lactoferrin exerts its antimicrobial effect in two ways: binding of iron and disruption of the integrity of the cell membrane [21].

α- Lactalbumin (LA) is a polypeptide with a molecular weight of 14 kDa, which is synthesized by liver cells and makes up about 20% of breast milk serum protein [22]. It contains a calcium ion and is characterized by a high content of cysteine, tryptophan, and lysine. LA plays a key role in the biosynthesis of lactose and has a three-dimensional structure like lysozyme. The high content of tryptophan, a precursor of serotonin, is responsible for its anti-stress effect. The LA-based derivative has antitumor [23] and antimicrobial effects [24].

Lysozyme (LC) or muramidase is a polypeptide with a molecular weight of 15 kDa that accounts for 6% of the total amount of whey protein in breast milk, synthesized by monocytes, PMN, and epithelial cells [25-26]. It has been established that LC is a coactivator of IgA, and its antimicrobial action against fungal and bacterial cells is realized by the destruction of glycosidic bonds of cell wall polysaccharides and damage to the cytoplasmic membrane [27]. Besides, positively charged lactoferrin acts synergistically with lysozyme *in vivo*, which leads to the binding and forming of a strong complex with a negatively charged lipopolysaccharide of gram-negative bacteria [28].

Lactoperoxidase (LP) is a monomeric heme-containing glycoprotein with a molecular weight of 80 kDa, which is synthesized by mammary gland cells [7]. The concentration of LP in whey protein is less than 0.01% [29]. LP catalyzes the oxidation of thiocyanate with hydrogen peroxide to hypothiocyanite, which exhibits antimicrobial properties [30]. Thus far, no direct antimicrobial activity of LP without association with thiocyanate has been detected [31].

The detrimental effect of the AMPs mentioned above on various microorganisms has been studied for a long time by the inoculation method. However, the antimicrobial effect of their combination has not been studied thoroughly before. For example, it has been previously shown that lactoferrin and lactoperoxidase/thiocyanate exhibit a synergistic effect against *C. albicans* when acting together [32]. However, the effect of lactoferrin and lysozyme on the same yeast species did not reveal the presence of synergy [33].

Thus, the aim of the present research was to study the combined effect of LF, LA, LC, and LP on the cells of *Candida albicans, Staphylococcus aureus,* and *Escherichia coli in vitro*.

MATERIALS AND METHODS

Candida albicans No. 927 (collection of the Mechnikov Research Institute for Vaccines and Sera) was cultivated on a glucose-peptone-yeast extract medium, *Staphylococcus aureus* Wood 46 - on GRM agar, *Escherichia coli* M 17 - on nutrient agar until the end of the exponential growth phase.

To assess the activity, we used preparations of lactoferrin (LF), lactalbumin (LA), and lactoperoxidase (LP) obtained by preparative ion-exchange chromatography from pools of breast milk from healthy mothers (Lactobio, Russia); lysozyme (LC) was derived from lyophilized egg (BioChemica qualification, AppliChem, USA).

To study the dose-dependent effect, each AMP was tested in the concentration range from 2.5 mg/ml to 20 mg/ml. To assess the combined effect of the tested substances, we added each of them to the mixture in the concentration of 5 mg/ml. In this case, the cumulative effect of preparations on a given microorganism was assessed together with their individual effects within one series of experiments. The total antimicrobial activity was calculated as the sum of the activities of each substance alone.

Antimicrobial activity was assessed by spectrophotometry [34]. To do this, 300 µl of a substance solution in saline was mixed with 50 µl of a suspension of microorganism cells prepared at the rate of 1 microbiological loop with a diameter of 1 mm in 50 µl of saline; the control sample

contained 300 μl of saline. The samples were incubated for 2 h at 32 °C on a shaker, and centrifuged for 5 min at 16000 rpm. Then, the supernatant was removed, and 300 μL of a solution of bromocresol purple in phosphate buffer pH 4.6 was added to the sediments, incubated for 45 min at 32 °C, and centrifuged again. The sediments were checked by microscopy at a final magnification of 1750x (LOMO, Russia) and photographed with a Sony digital camera (Japan). From the supernatants, 50 μl was taken and mixed with 2.5 ml of phosphate buffer, pH 4.6. The optical density of the solutions was assessed on a Genesys 10SUV-Vis spectrophotometer (USA) at a wavelength of 440 nm in a 1 cm cuvette. The average value of three measurements was calculated for each sample. The activity was calculated as the ratio of the difference between the optical density of the control and experimental samples, referred to as the optical density of the control sample, and expressed as a percentage [35].

Statistical analysis was performed using Microsoft Excel 2010. The calculation of the Mann-Whitney coefficients, indicating the presence and absence of significance of differences between the indexes, was carried out using the automatic program [36].

RESULTS

The tested AMPs applied in the concentrations ranging from 2.5 mg/ml to 20 mg/ml demonstrated a dose-dependent antimicrobial effect for LF, LP, and LC taken alone (Table 1). The sediments of microbial cells exposed to these AMPs after staining with bromocresol purple were dark brown compared to light yellow control sediments. Microscopy of sediment samples with *C. albicans* showed that yeast cells under the influence of these AMPs were destroyed with the formation of colored vesicular debris; a typical result is shown by the example of LF (Fig. 1). Compared to LF, the LA did not show any antimicrobial activity, i.e., the cell sediments of all three species of microorganisms were stained in the same way as the control samples, and microscopy also did not reveal any differences from the control samples. The table shows that at low concentrations, LP was the most active against yeasts, and LC was the most active against bacteria, while at high concentrations, LC turned out to be the best among all three species of microorganisms taken for the experiments.

Further studies were carried out with LF, LP, and LC. The comparison of the action of AMPs combined in pairs showed the following results (Fig. 2). For

Table 1. The antimicrobial activity of AMPs.

Polypeptide	Microorganism	Antimicrobial activity of different concentrations of polypeptides, % (M±m)			
		2.5 mg/ml	5 mg/ml	10 mg/ml	20 mg/ml
Lactoferrin	*C. albicans*	6.8 ± 1.2	11.4 ± 0.2	26.7 ± 0.7	37.7 ± 0.6
	S. aureus	6.2 ± 0.8	13.2 ± 2.4	27.5 ± 0.4	32.5 ± 2.4
	E. coli	4.6 ± 0.3	14.4 ± 0.3	27.0 ± 2.6	39.5 ± 1.2
Lactalbumin	*C. albicans*	8.4 ± 1.0	6.9 ± 0.5	5.6 ± 1.1	7.6 ± 1.6
	S. aureus	-1.3 ± 1.5	-2.3 ± 1.0	-2.3 ± 0.4	-6.3 ± 0.6
	E. coli	0.2 ± 1.1	-1.9 ± 1.4	3.5 ± 0.1	2.2 ± 1.9
Lactoperoxidase	*C. albicans*	12.9 ± 0.4	14.7 ± 0.9	25.7 ± 1.6	38.9 ± 0.8
	S. aureus	1.0 ± 0.6	6.3 ± 1.8	19.7 ± 1.0	23.8 ± 1.0
	E. coli	6.3 ± 2.5	9.6 ± 0.8	24.6 ± 0.4	24.8 ± 0.6
Lysozyme	*C. albicans*	5.3 ± 0.8	19.4 ± 0.8	23.0 ± 0.5	62.4 ± 0.1
	S. aureus	17.9 ± 0.5	36.7 ± 1.3	41.6 ± 1.2	56.6 ± 1.8
	E. coli	12.7 ± 0.6	28.6 ± 0.1	31.6 ± 0.2	62.4 ± 0.5

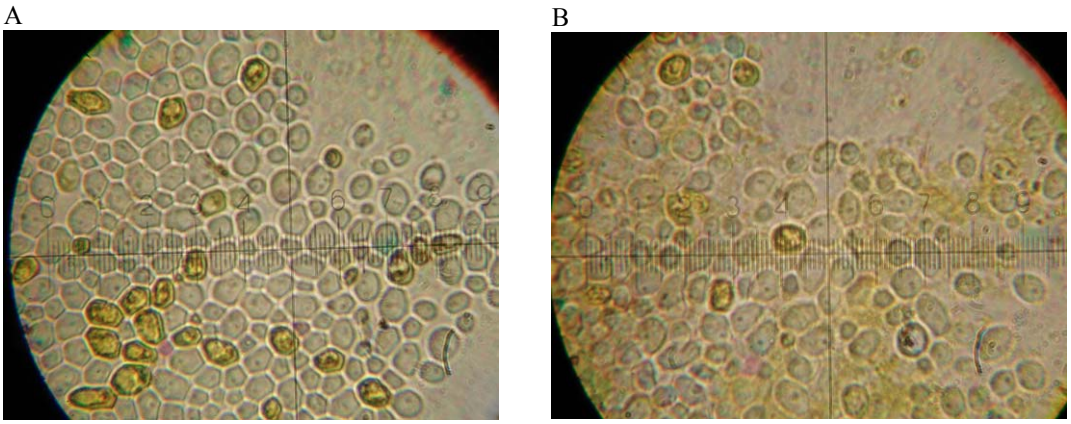

Fig. 1. The effect of lactoferrin on *C. albicans* cells (microphotograph): **A** - control cells (incubation with saline); **B** - experiment (incubation with lactoferrin at a concentration of 10 mg/ml).

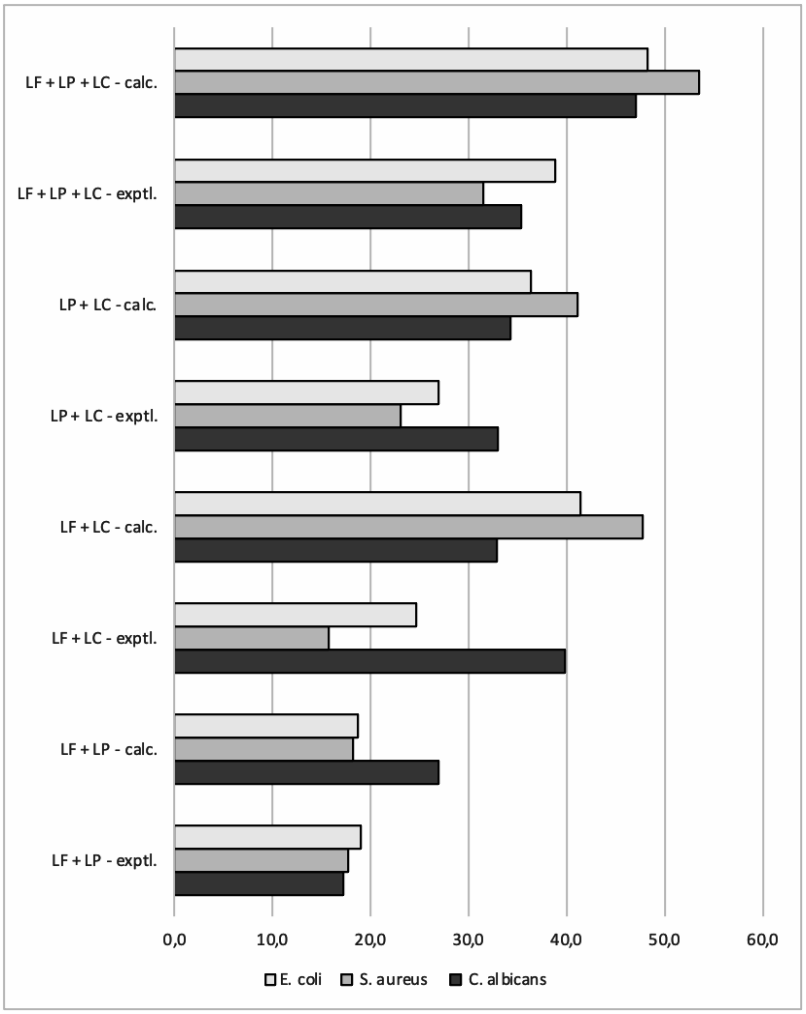

Fig. 2. The comparison of the experimental (exptl.) and calculated (calc.) values of the microbicidal activity of lactoferrin (LF), lysozyme (LC) and lactoperoxidase (LP) with their combined effect on microorganisms.

the pair of LF and LP, the experimental and calculated values of the total activity turned out to be almost the same against both types of bacteria, while the calculated activity against *C. albicans* was 1.6 times higher than the experimental one ($p \leq 0.01$). That is, in the case of yeasts, the combined action of LF and LP exhibited an antagonistic effect.

For the pair of LF and LC, the sum of the calculated activities significantly exceeded the sum of the experimental ones; for *E. coli*, it was 1.7 times ($p \leq 0.01$), and for *S. aureus*, it was 3 times ($p \leq 0.01$), i.e., significant mutual inhibition was noted. On the contrary, there was insignificant synergy for *C. albicans,* i.e., the experimental total activity was 1.2 times higher than the calculated one ($p > 0.05$).

The combination of LP and LC exhibited an antagonistic effect; it was 1.4 times less for *E. coli* ($p \leq 0.01$) and 1.8 times less for *S. aureus* ($p \leq 0.01$). However, for *C. albicans*, these indexes practically did not differ.

When we used combinations of all three AMPs, LF, LC, and LP, the calculated activities significantly exceeded the experimental ones for all species of microorganisms involved: 1.2 times for *E. coli* ($p \leq 0.01$), 1.7 times for *S. aureus* ($p \leq 0.01$), and 1.3 times for *C. albicans* ($p \leq 0.01$). Thus, there was a mutual inhibition of activity noted with the simultaneous action of AMP preparation on all three species of tested microorganisms.

To sum up, for bacteria, in all variants, except for the pair of LF and LP, an antagonistic effect was observed, which was most pronounced for the pair of LF and LC. At the same time, for yeast, this effect was observed only in the LF and LP variants and in the case of the action of all three preparations.

DISCUSSION

Human breast milk contains a set of AMPs, such as LF, LP, LC, as well as cathelicidin, defensins, etc. [37]. The bactericidal and fungicidal effect of LP in combination with hydrogen peroxide and thiocyanate on microbial cells (lactoperoxidase system) estimated by the inoculation method has been previously shown [38]. The spectrophotometry method allows assessing quickly the microbicidal effect associated with the destruction of the cytoplasmic membrane and cell wall [34]. In the present study, for the first time, the antimicrobial activity of LP against microbial cells was established using this method, i.e., out of the lactoperoxidase system. It has been shown that LF, LP, and LC destroy the cells of microorganisms with the formation of vesicles. Also, no direct antimicrobial activity was found in LA, despite such effect exhibited by LA when used in combination with oleic acid [39]. The fungistatic effect of human LF on *C. albicans* cells was shown earlier by the routine culture method, and the minimum inhibitory concentration of LF of about 6 mg/ml was determined [40, 41]. The simultaneous action of LF and LC on the same yeast species was also studied by the inoculation method, and the absence of synergy was shown [33]. However, in the present study, the insignificant synergy in the action of LF/LC on *C. albicans* has been demonstrated by spectrophotometry. Previously, a synergistic antimicrobial effect of LF and LC against *S.epidermidis* was shown by cultural methods [42]. Our study shows that the combination of LF/LC has an antagonistic effect on *S.aureus* and *E.coli.* The combination of all three AMPs (LF/LP/LC) was previously used to prepare compositions of toothpaste, and this preparation was used in combination with thiocyanate and hydrogen peroxide [43]. The author of the review noted that LP retained its activity *in vivo*, producing antimicrobial hypothiocyanite, which cannot be assumed regarding the activity of LF and LC. Another *in vivo* study showed the presence of six different stable combinations of LF, LC, peroxidase, and immunoglobulin A in different ratios in saliva [44]. The authors explained that these combinations may be a product of independent variations in the secretory activity of acinar and intercalated duct cells.

CONCLUSION

The investigation of the mutual influence of LF, LP, and LC *in vitro* was carried out for the first time in this study, and it was shown that the combined action of all three preparations exerted an antagonistic effect; i.e., the total activity was significantly lower than the sum of the activities of individual preparations. Possibly, such a situation also occurs *in vivo* in cases when the synthesis of any of the components of the system is interchanged.

The data obtained are necessary for further research focusing on the elaboration of new recipes to enrich milk formulas for artificial feeding of babies, which may be more nutritious and beneficial.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST STATEMENT

The author declares no conflict of interest, financial or otherwise.

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