Journal of Bacteriology and Parasitology

Anacarso, et al., J Bacteriol Parasitol 2014, 5:5
DOI: 10.4172/2155-9597.1000201

Research Article Open Access

Amoebicidal Effects of Three Bacteriocin like Substances from Lactic Acid Bacteria against *Acanthamoeba Polyphaga*

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Recieved date May 15, 2014; **Accepted date** September 24, 2014; **Published date** September 27, 2014

Abstract

We investigated the antiamoebic activity of three Bacteriocin Like Substances (BLS 39, BLS GS 54, BLS GS 16) produced by Lactic Acid Bacteria (LAB). The crude bacteriocins showed an amoebicidal effect against *Acanthamoeba polyphaga*, but with differences. BLS 39, produced by *Lactobacillus pentosus*, determined a prompt and progressive decrease of viable amoebal cell count, up to the end of the experiment (144 h), where the trophozoites were not detectable. A killing effect, but after a more prolonged contact time, was observed for BLS GS 54, produced by *Lactobacillus paraplantarum*, whereas the bacteriocin produced by *Lactobacillus plantarum* GS16 showed the lowest toxicity for *A. polyphaga*. For BLS GS 16 the maximum percentage of reduction in trophozoites count (45%) was obtained after 144 h, value much lower when compared to BLS GS54 and BLS 39, that showed values of 44,60% and 52,60% after only one hour of contact, with a maximum of 98% and 100% of non-viable cells, respectively, after 144 h. Morphological changes of the *A. polyphaga* cells as swollen cells, roundness and cellular lysis, were already observed after the first hours of contact with BLS and, at the end of the experiment, most of the cells were colored (blue), indicating their death. Currently there isn't evidence of BLS produced by LAB active against amoebas. In this study we have shown that all the three BLS secreted by the Lactic Acid Bacteria are endowed with amoebicidal effect against *Acanthamoeba polyphaga*, killing the protozoan with different effectiveness and at different times of contact.

Keywords: Amoebicidal, Bacteriocin like substances, Lactic acid bacteria, *Acanthamoeba polyphaga*, Lactobacilli

Introduction

Acanthamoeba is a ubiquitous free-living protozoan that can exit as motile trophozoites and form cysts in response to adverse environmental conditions [1,2]. In either the trophic or the cyst stage, the organisms belonging to this genus have a wide distribution in nature, and they can be isolated from soil, water and other environmental samples. Several species of *Acanthamoeba*, as *A. castellanii, A. culbertsoni, A. hatchetti, A. healyi, A. polyphaga, A.* rhysodes, *A. astronyxis, A. divionensis*, are known to cause disease in humans, then *Acanthamoeba* is the causative agent of an insidious, chronic and mostly fatal disease, granulomatous amoebic encephalitis (GAE), especially in immunocompromised patients [3]. Amoebic keratitis, caused by *Acanthamoeba*, affects mainly the wearers of contact lenses [1,4], and skin infections, nasopharyngeal and systemic diseases are reported [5]. *Acanthamoeba* also can serve as hosts for endocytobionts, representing a significant reservoir for environmental pathogen/opportunistic microorganisms (*Legionella, Aeromonas hydrophila*) and food-borne pathogens (*Listeria monocytogenes, Salmonella enterica serovar Enteritidis*, *Yersinia enterocolitica*), with important implications for human health [6-8]. The medical therapy is often difficult because, under unfavorable conditions, *Acanthamoeba* trophozoites undergo a cellular differentiation process (encystment), resulting in the formation of double-walled cysts with increased resistance to antimicrobial agents and the therapy has to be maintained for a long period and not always with a favorable outcome [9]. In recent years new strategies have been developed, which involve the use of various natural substances. There are several evidences of amoebicidal activity of essential oils obtained from *Euphorbiaceae* plant, [10], *Pterocaulon polystachyum* [11,12], *Allium sativum* extract [13], Propolis [14], and BLS (Bacteriocin Like Substances) [11,15-17]. BLS are ribosomally synthesized antimicrobial peptides, generally small (<10 KDa), amphipathic and cationic peptide of variable length and structure. The antimicrobial peptide synthesized from LAB (Lactic Acid Bacteria), also referred as bacteriocins, are active against other closely related bacteria (narrow spectrum), or against other not genetically related genera (broad spectrum) [18,19]. Bacteriocins produced by LAB, recently, have attracted much attention because of their GRAS status and potential use as natural additives directly in food or in food-packaging [20,21], and as potential therapeutic agents [22]. Some peptides have a broad spectrum of activity against various microorganisms, comprising Gram-positive and Gram-negative bacteria, protozoa, yeast, viruses [15]. In the present study we have assessed "in vitro" the amoebicidal activity of three BLS produced by our isolated LAB against *Acanthamoeba polyphaga*, determining the cell viability or other morphological changes at different times, up to the end of the investigation (144 h).

Materials and Methods

Microorganisms and conditions of growth

Acanthamoeba polyphaga ApUP strain used was isolated from a person affected by Acanthamoeba keratitis, kindly supplied by the Department of Microbiology-University of Parma. The Acanthamoeba ApUP used in our previous studies [8,23,24], belonging to the T4 genotype, was maintained in axenically conditions as monolayers in 25

cm^2 tissue culture flasks (Sarstedt, USA), and cultured in axenically with peptone-yeast-glucose broth (PYG, OXOID Milan, Italy) at 30°C. For testing, trophozoites in exponential phase growth (72-96 h) were washed twice in phosphate-buffered saline (PBF) buffer (pH 7.2) and re-suspended in the same media (PYG).

The bacteriocins producers were *Lactobacillus pentosus* 39, isolated in our previous work (7) and *Lactobacillus paraplantarum* GS54, and *Lactobacillus plantarum* GS16, isolated from ham steak and identified by PCR. The primers used for this purpose were ParaPla-for (5'-CAG TGG CGC GGT TGA TAT-3') and P-rev (5'-TCG GGA TTA CCA AAC ATC AC-3') for *L. paraplantarum*; Plant-for (5'-atc atg att tac att tga ctg-3') and LovLac2-rev (5'-CGA CGA CCA TGA ACC ACC TGT-3') for *L. plantarum*. The reactions were performed with the following conditions: 2 min at 94°C (1 min at 92°C, 1 min at 45°C, 1 min at 72°C) for 30 cicles, 10 min at 72°C and the results were highlighted by 1% gel electrophoresis. The producers were cultivated in PYG (OXOID Milan, Italy) anaerobically at 30°C for 48 h to minimize the formation of hydrogen peroxide [25]. Cells were harvested by centrifugation at 10,000x g for 15 min at 4°C, and the supernatant was filtered through 0.22-µm membranes (Millipore, Milan, Italy) to obtain the crude bacteriocins (BLS 39, BLS GS 54, BLS GS 16, respectively). The antimicrobial titer of the three bacteriocins were evaluated in terms of arbitrary units per millilitre (AU ml-1) and these were used, after appropriate diluitions, all with the same concentration of 800 AU/ml.

Amoebicidal activity

The experiments were performed in sterile 96-well plate with the flat bottom. Wells were seeded with 200 µl of the previous *A. polyphaga* suspension in PYG (about 5×10^4/well) and incubated for 24 h at 30°C to allow the attach of the trophozoites to the well surfaces. Once confluent, the not adherent amoebae were removed by washing the wells once with PAS (Page's Amoebic Saline: 0.120 g l^{-1} NaCl, 0.004 g l^{-1} $MgSO_4\times7$ H_2O, 0.004 g l^{-1} $CaCl_2\times2$ H_2O, 0.142 g l^{-1} Na_2HPO_4, 0.136 g l^{-1} KH_2PO_4). Afterwards, the wells were filled with 100 microliters of the three crude bacteriocins, and the cell viability was evaluated by Trypan blue assay (Sigma-Aldrich, Milan, Italy) after 1, 4, 24, 48, 72 and 144 hours. At the established times, each well was filled with a Trypan blue solution, to have a concentration for each well of 0.4% and the cell-count was done in a Bürker chamber under an inverted microscope (200x magnification), counting separately both the blue (death) and the white (live) cells. Wells with the same concentration of amoebae but without BLS, and incubated in the same conditions, were used as negative control.

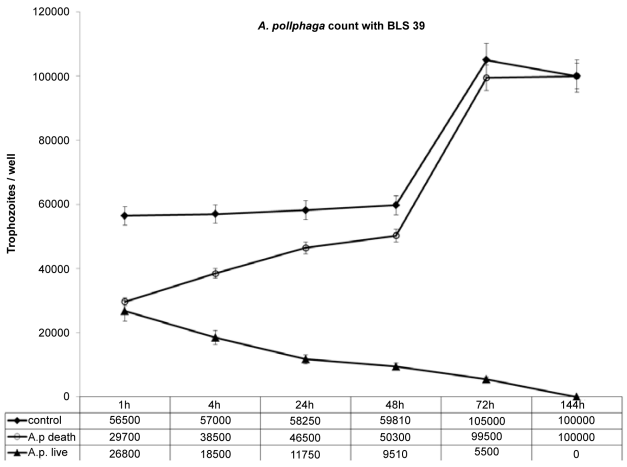
All the experiments were carried out in triplicate, and the results reported as arithmetic mean of the three determinations. The standard deviation was reported as error bars. The rates of decline of amoebal live cells and increase of amoebal dead cells were analyzed with a t-test for paired data. Statistical probability equal to or less than 0.05 was considered significant.

Results and Discussion

In the present study, natural peptidic substances (BLS) produced by three LAB, were evaluated for antiamoebic activity.

The producers LABs were cultivated in PYG, the same medium for amoeba, and the crude bacteriocins (BLS 39, BLS GS 54, BLS GS 16), obtained by filtration through 0.22-µm membranes of LAB cell-free supernatant. The negative control was the only PYG medium. All three substances tested showed amoebicidal effects against *Acanthamoeba polyphaga*, but with differences.

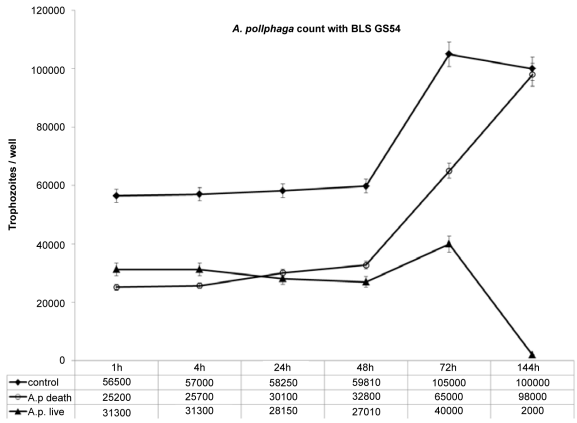
In the Figures 1-3 we reported the counts of both the dead and the live cells, in comparison with the control. In particular, BLS 39 (Figure 1) showed a strong amoebicidal activity, determining a prompt and progressive decrease of viable amoebal cell count (p=0.0049 control vs live cells), up to the end of the experiment (144 h), where the trophozoites were not detectable. At the same time, an immediate and constant in time increase of dead amoebal cells has been observed (p=0.027 control vs dead cells). A killing effect, but after a more prolonged contact time, was observed for BLS GS 54 (Figure 2). In this case, *A. polyphaga* trophozoites count remained nearly constant until 48 h incubation, and then rapidly declined up to the end of the experiment, while, the opposite trend was observed for the dead cell count (p=0.0121 control vs live cells; p=0.0039 control vs dead cells). With regard to the amoebicidal activity of bacteriocin produced by *L. pentosus* GS16, the substance resulted in a lower toxicity for *A. polyphaga*, when compared to the other two BLS (Figure 3), with a trend almost similar to the control, from which it differs only after 144 hours. BLS GS 16 showed a reduction in viable cell count slightly more than what can be considered a physiological decrease (p=0.0186 control vs live cells) but its killing activity was however confirmed by the progressive increase in the number of dead cells (p=0.00020 control vs dead cells). This data is easily visible in Figure 4, which shows the cell mortality data of *A. polyphaga*, obtained by Trypan blue assay. We can observe that for BLS GS 16 the maximum percentage of reduction in trophozoites count, (45%), was obtained only after 144 h, value much lower when compared to BLS GS54 (p=0.0105) and BLS 39 (p=0.0033). These last, in fact, killed a percentage of trophozoites of 44,60% and 52,60% after only one hour of contact, with a maximum of 98% and 100% of non-viable cells, respectively, after 6 days (144 h).



	1h	4h	24h	48h	72h	144h
control	56500	57000	58250	59810	105000	100000
A.p death	29700	38500	46500	50300	99500	100000
A.p. live	26800	18500	11750	9510	5500	0

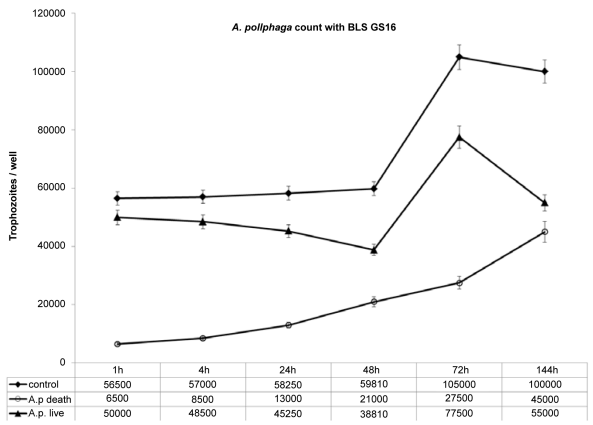
Figure 1: Survival/death trend of *A. polyphaga* trophozoites in wells added with BLS 39

Lastly, Figures 5 and 6 show the cell morphology of *A. polyphaga*, evaluated using an inverted microscope at all the times indicated before (1 h, 4 h, 24 h, 48 h, 72 h, 144 h). The protozoan cells showed morphological changes, already after the first hours of contact (1 h and 4 h), (data not shown), such as swollen cells, roundness and cellular lysis but no cysts were detected.



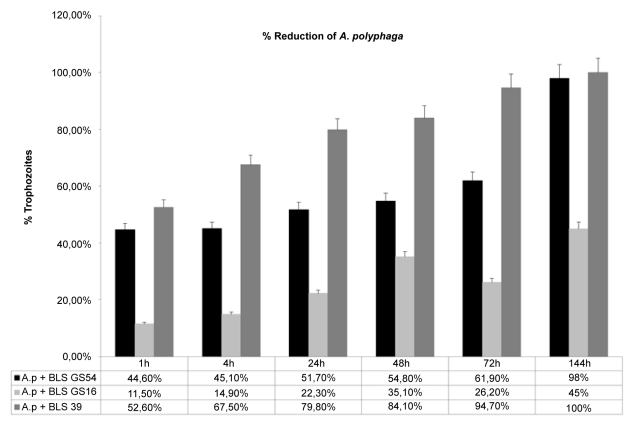
	1h	4h	24h	48h	72h	144h
control	56500	57000	58250	59810	105000	100000
A.p death	25200	25700	30100	32800	65000	98000
A.p. live	31300	31300	28150	27010	40000	2000

Figure 2: Survival/death trend of *A. polyphaga* trophozoites in wells added with BLS GS 54



	1h	4h	24h	48h	72h	144h
control	56500	57000	58250	59810	105000	100000
A.p death	6500	8500	13000	21000	27500	45000
A.p. live	50000	48500	45250	38810	77500	55000

Figure 3: Survival/death trend of *A. polyphaga* trophozoites in wells added with BLS GS 16



	1h	4h	24h	48h	72h	144h
A.p + BLS GS54	44,60%	45,10%	51,70%	54,80%	61,90%	98%
A.p + BLS GS16	11,50%	14,90%	22,30%	35,10%	26,20%	45%
A.p + BLS 39	52,60%	67,50%	79,80%	84,10%	94,70%	100%

Figure 4: Percentage of growth reduction of *A. polyphaga* trophozoites at different times of incubation after exposure to the three Bacteriocin Like Substances

These changes were more evident after 24 h (Figure 5) and at the end of the experiment (Figure 6), where most of the cells was colored (blue), indicating their death. However, to ensure that the Trypan blue has really colored dead cells, the plates were maintained under observation for the next week at the end of the experiment, to assess that there were no variations on the cell count and cell shape displaying a residual vitality.

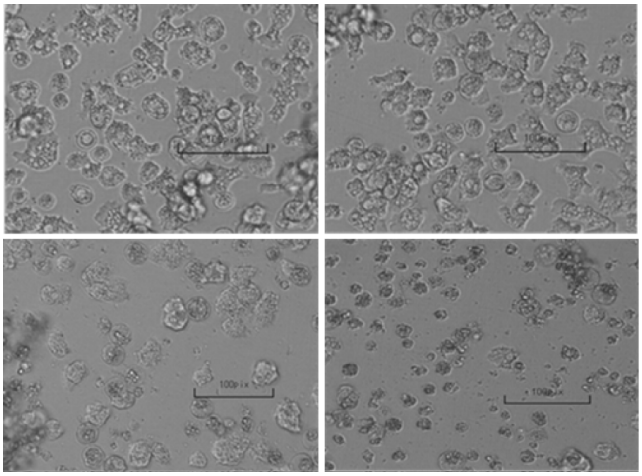


Figure 5: Cellular changes observed by inverted microscope, after 24 h incubation of *A. poliphaga* cells added with PYG medium (a control), BLS GS16 (b), BLS GS54 (c) and BLS 39 (d).

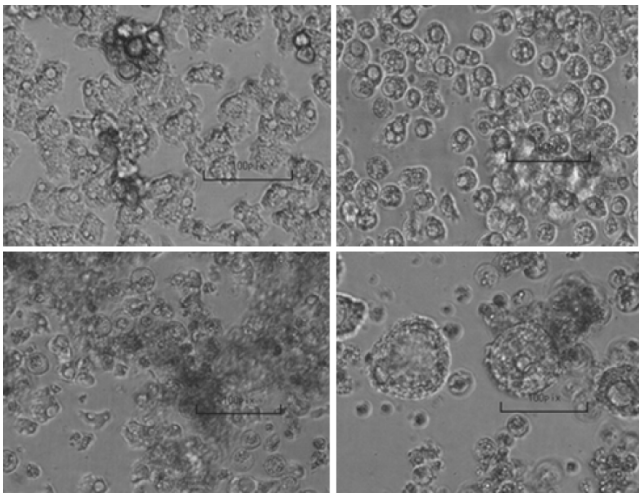


Figure 6: Cellular changes observed by inverted microscope, after 144 h incubation of *A. poliphaga* cells added with PYG medium (a control), BLS GS16 (b), BLS GS54 (c) and BLS 39 (d).

Other peptidic substances were tested against various protozoa and have been reported by other Authors. Benitez showed the inhibition of *A. polyphaga* by BLS from *Bacillus amyloliquefaciens* [15]. Sacramento et al. reported the ability of an alpha-helical and beta-hairpin antimicrobial peptides to interfere with both growth and cell permeability of *Acanthamoeba castellanii* [26]. Previously, Lebradi et al. established the activity of amoebicin from *Bacillus licheniformis*

against *Naegleria fowleri* [17]. Currently there isn't evidence of BLS produced by LAB active against amoebas. In this study we have shown that all the three BLS secreted by the Lactic Acid Bacteria are endowed with amoebicidal effect against *Acanthamoeba polyphaga*, killing the protozoan with different effectiveness and at different times of contact. BLS 39, in particular, resulted the best active substance, with statistical significant differences compared to the other two bacteriocins employed in the study.

Acanthamoeba polyphaga is very difficult to eradicate, because some drugs used for this purpose haven't amoebicidal effects, but only amoebistatic and they are often poorly tolerated by the host cells, particularly if used for a long period [26].

The development of new strategies will be necessary in future to help fight infections caused by this protozoan. Recent investigations have focused the attention on the amoebicidal effects of natural agents from plants and of bacterial origin [11,12,14-17], but no substances extracted from LAB have never been used for this purpose. Lactic Acid Bacteria have already received a special attention in food and environmental fields, and the outcome achieved with the use of either their bacteriocins or the bacteriocin-producing LAB to control pathogenic bacteria [7,22,27] has opened broader fields of application. New generation of drugs, including natural anti-protozoarian substances, could be developed for the treatment of parasitic diseases, and with this purpose three different BLS by LAB, all endowed with amoebicidal effects, were tested in this preliminary investigation. The results reported in this study are preliminary but encouraging, opening a possible path for the use of these substances for other investigations with future clinical purposes. Further studies, based on the mode of action of the purified amoebicidal bacteriocins and the determination of the degree of effectiveness, and the lack of toxicity of these peptidic compound, are necessary to better define their future impact on public health in humans.

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