BACTERIOCIN PRODUCTION BY *LACTOBACILLUS PLANTARUM* AMA-K ISOLATED FROM AMASI, A ZIMBABWEAN FERMENTED MILK PRODUCT AND STUDY OF THE ADSORPTION OF BACTERIOCIN AMA-K TO *LISTERIA* SP.

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ABSTRACT

Bacteriocin AMA-K produced by *Lactobacillus plantarum* AMA-K inhibits the growth of *Enterococcus* spp., *Escherichia coli, Klebsiella pneumoniae* and *Listeria* spp. Growth of strain AMA-K in BHI, M17, soy milk and molasses was similar to growth in MRS. The effect of organic nitrogen sources, carbohydrates, glycerol, K_2HPO_4 and KH_2PO_4, $MgSO_4$, $MnSO_4$, tri-ammonium citrate, Tween 80, vitamins and initial pH on bacteriocin AMA-K was determined. The mode of action of bacteriocin AMA-K was studied. The effect of bacteriocin AMA-K to actively growing *Listeria innocua* LMG13568, *L. ivanovii* subsp. *ivanovii* ATCC19119 and *L. monocytogenes* ScottA was determined. Adsorption of bacteriocin AMA-K to target cells at different temperatures, pH and in presence of Tween 20, Tween 80, ascorbic acid, potassium sorbate, sodium nitrate and sodium chloride were studied. Bacteriocin AMA-K shares high homology to pediocin PA-1.

Key words: Bacteriocin AMA-K; *Lactobacillus planatrum*; Amasi

INTRODUCTION

Amasi is a traditional fermented milk product consumed in different regions of Southern African, including Zimbabwe, South Africa and Lesotho. The product is an unsweetened curd with a consistency slightly thicker than yoghurt and with a pH between 3.6 and 4.2. Although normally consumed with thick corn-meal porridge, Amasi is also consumed between meals with ground sorghum, similar to muesli.

Traditionally Amasi is produced from unpasteurised bovine (cow's) milk and is allowed to ferment spontaneously in an earthenware (clay) pot or gourd ("calabash") for two to three days at ambient temperature. The microbial flora responsible for the fermentation is derived from the air, raw milk and walls of the containers. After coagulation, the whey is drained through a plugged hole at the bottom of the container.

Fermentation is dominated by lactic acid bacteria (*Lactobacillus plantarum*, *Lactobacillus helveticus*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc lactis*, *Leuconostoc citreum*, *Leuconostoc mesenteroides* subsp.

dextranicum, *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus casei* subsp. *casei* and *Lactobacillus casei* subsp. *pseudoplantarum*) typically in the order of 10^8 CFU/ml (4).

Lactic acid bacteria are known for their production of antimicrobial compounds, including bacteriocins or bacteriocin-like peptides (10). Bacteriocins of LAB are defined as ribosomally synthesized proteins or protein complexes usually antagonistic to genetically closely related organisms (10). In previous work the characterization of a bacteriocin produced by *Lactobacillus plantarum* AMA-K was described (43). Bacteriocins are generally low molecular weight proteins that gain entry into target cells by binding to cell surface receptors. Their bactericidal mechanism vary and may include pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rDNA, and inhibition of peptidoglycan syntesis (10,15).

In recent papers (23,29), specific environmental conditions, including those found in food, have been studied to determine their effect on the production of bacteriocins. Bacteriocin production changes dramatically upon altering of

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environmental conditions and optimum production may require a specific combination of environmental parameters (22). Little is known about the interactions these factors have on the production of a bacteriocin, especially in a complex food environment.

Apart from studies conducted on the effect of nitrogen and carbon sources on the production of plantaricin ST31 (37), plantaricin 423 (46), plantaricin UG1 (13), plantaricin KW30 (19), plantaricin-149 (18), plantaricin S (16), plantaricin ST13BR (44) and plantaricin A (8), little is known about the growth conditions required for optimal production of these bacteriocins. Studies conducted on bacteriocins from other lactic acid bacteria, e.g. pediocin AcH (5), pediocin PD-1 (30), enterocin 1146 (32), enterocin AS-48 (2), enterocin P (14), sakP (1), and bacteriocins produced by *Leuconostoc mesenteroides* L124 (26) have shown that production is often regulated by growth pH and temperature. In some cases, higher bacteriocin activity has been recorded at sub-optimal growth conditions (1,3,6,11,20,21,27,28,32,33,37).

The aim of this study was to determine the conditions needed for optimal production and study some aspects of mode of action of bacteriocin AMA-K produced by *L. plantarum* AMA-K isolated from Amasi.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Strain AMA-K, isolated from Amasi produced in Gwanda, Kafusi, in the South-Western region of Zimbabwe, was classified as *L. plantarum* based on phenotypic and genotypic characteristics (43). The strain was cultured in MRS medium (Biolab, Biolab Diagnostics, Midrand, SA) at 30ºC and stored at -80ºC in spent MRS broth, supplemented with 15% (v/v) glycerol. MRS broth (Biolab) was used in all experiments, except growth optimization, in which case MRS broth (9) was modified as indicated.

Bacteriocin bioassay

Bacteriocin screening was performed by using the agar-spot-test method (34). Correction of the cell-free supernatant to pH 6.0 with 1M NaOH prevented the inhibitory effect of lactic acid. Antimicrobial activity was expressed as arbitrary units (AU/mL), calculated as $a^b \times 100$, where "a" represents the dilution factor and "b" the last dilution that produces an inhibition zone of at least 2mm in diameter. Activity is expressed per mL by multiplication with 100. One AU is defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition (34). *Listeria innocua* LMG13568 was used as indicator strain.

Cell-free supernatant containing bacteriocin AMA-K was incubated at 25ºC for 68h and at regular intervals bacteriocin activity was determined as described before.

Bacteriocin production in different growth media and at different initial growth pH

An 18h-old culture of strain AMA-K was inoculated (2%, v/v) into MRS broth (Biolab), BHI broth, M17 broth (Merck), soy milk (10%, w/v, soy flour) and molasses (10%, w/v), respectively. Incubation was at 30ºC and 37ºC, respectively, without agitation, for 25h. Samples were taken every hour and examined for bacterial growth (OD at 600 nm), changes in culture pH, and production of bacteriocins (AU/mL). The agar-spot-test method was used, with *L. innocua* LMG13568 as target organism.

In a separate experiment, the effect of initial medium pH on the production of bacteriocin AMA-K was determined. Volumes of 300 mL MRS broth were adjusted to pH 4.5, 5.0, 5.5, 6.0 and 6.5, respectively, with 6M HCl or 6M NaOH and then autoclaved. Each flask was inoculated with 2% (v/v) of an 18h-old culture of *L. plantarum* AMA-K and incubated at 30ºC for 24h, without agitation. Changes in culture pH and production of bacteriocin AMA-K, expressed as AU/mL, were determined every hour as described elsewhere. All experiments were done in triplicate.

Effect of medium composition on bacteriocin production

L. plantarum AMA-K was grown in 10 mL MRS broth (Biolab) for 18h at 30ºC, the cells harvested by centrifugation (8000xg, 10min, 4ºC), and the pellet re-suspended in 10 mL sterile peptone water. Four ml of the cell suspension was used to inoculate 200 mL of the following media: (a) MRS broth (9), without organic nutrients, supplemented with tryptone (20.0g/L), meat extract (20.0g/L), yeast extract (20.0g/L), tryptone (12.5g/L) plus meat extract (7.5g/L), tryptone (12.5g/L) plus yeast extract (7.5g/L), meat extract (10.0g/L) plus yeast extract (10.0g/L), or a combination of tryptone (10.0g/L), meat extract (5.0g/L) and yeast extract (5.0g/L), respectively; (b) MRS broth, i.e. with 20.0g/L D-glucose; (c) MRS broth without D-glucose, supplemented with 20.0g/L fructose, sucrose, lactose, mannose, maltose and gluconate, respectively; (d) MRS broth with 5.0 to 50.0g/L glucose as sole carbon source; (e) MRS broth with 2.0 to 20.0g/L K_2HPO_4, 2.0 to 20.0g/L KH_2PO_4 or combination of 2.0g/L K_2HPO_4 and 2.0g/L KH_2PO_4; (f) MRS broth supplemented with 1.0 to 50.0g/L glycerol; (g) MRS broth without $MgSO_4$; (h) MRS broth without $MnSO_4$; (i) MRS broth without or supplemented with 5.0g/L and 10.0g/L tri-ammonium citrate; and (j) MRS broth without Tween 80 or supplemented with 0.5 to 2.0g/L.

In a separate experiment, the vitamins cyanocobalamin (Sigma, St. Louis, Mo.), L-ascorbic acid (BDH Chemicals Ltd), thiamine (Sigma) and DL-6,8-thioctic acid (Sigma) were filter-sterilised and added to MRS broth at 1.0 mg/mL (final concentration). All cultures were incubated at 30ºC for 24h. Activity levels of bacteriocin AMA-K were determined as described elsewhere. All experiments were done in triplicate.

Cell lysis

In a separate experiment, 20 mL cell-free supernatant containing bacteriocin AMA-K (12800AU/ml, pH 6.0) was filter-sterilized (0.20 μm, Minisart®, Sartorius) and added to 100 ml 3-h-old cultures ($OD_{600} = 0.1 - 0.2$) of *L. innocua* LMG13568, *Listeria monocytogenes* ScottA and *Listeria ivanovii* subsp. *ivanovii* ATCC19119, respectively. Incubation was on BHI broth (Biolab) at 37ºC. Optical density readings were recorded at 600nm, hourly for 12h. The experiment was repeated with stationary-phase cells.

Adsorption of bacteriocin AMA-K to target cells

Adsorption of bacteriocin AMA-K to target cells was performed according to the method described by Yildirim *et al.* (47). The target strains (*L. innocua* LMG13568, *L. monocytogenes* ScottA and *L. ivanovii* subsp. *ivanovii* ATCC19119) were grown overnight in BHI broth at 37ºC and then centrifuged (8000x*g*, 15 min, 4ºC). Cells were washed twice with sterile 5mM phosphate buffer (pH 6.5) and re-suspended in the same buffer to OD at 600 nm equal to 1.0. The pH was adjusted to 6.5 with sterile 0.1M NaOH. Each cell suspension was mixed with an equal volume bacteriocin AMA-K (12800AU/ml, pH 6.5) and incubated at 37ºC for 1h. After removal of cells (8000x*g*, 15 min, 25ºC), the activity of unbound bacteriocin AMA-K in the supernatant was determined as described before. All experiments were done in duplicate.

The percentage adsorption of bacteriocin AMA-K to target cells was calculated according to the following formula:

$$\% \text{ adsorption} = \left[100 - \frac{AU/mL_1}{AU/mL_2} \right] \times 100$$

AU/mL$_1$ refers to the bacteriocin activity after treatment; *AU/mL$_0$* refers to the original (before treatment) activity.

Effect of pH and temperature on the adsorption of bacteriocin AMA-K

Bacteriocin AMA-K was added to *L. innocua* LMG13568, *L. monocytogenes* ScottA and *L. ivanovii* subsp. *ivanovii* ATCC19119, as described elsewhere, and incubated for 1h at 4, 15, 30, 37 and 45ºC, respectively (pH 7.0), and at 37ºC at pH 3.5, 5.5 and 7.0. Cells were harvested (8000x*g*, 15 min, 25ºC) and the pH of the cell-free supernatant adjusted to 6.0 with sterile 1M NaOH. Bacteriocin activity in the supernatant was determined as described before. The experiments were done in duplicate.

Effect of inorganic salts and organic compounds on adsorption of bacteriocin AMA-K to target cells

Cells of *L. innocua* LMG13568, *L. monocytogenes* ScottA and *L. ivanovii* subsp. *ivanovii* ATCC19119 were treated with 1% (m/v) Tween 20, Tween 80, NaCl, ascorbic acid, potassium sorbate and sodium nitrate. The pH of all samples were adjusted to 6.5 with 1 M NaOH or 1 M HCl. Bactericoin AMA-K was added to the treated cells, as described before, and incubated for 1h at 37ºC. The cells were harvested (8000x*g*, 15 min, 25ºC) and the activity of bacteriocin AMA-K in the cell-free supernatant determined as described before. The experiments were done in duplicate.

Identification of genes encoding bacteriocin production

DNA was isolated according to the method of Dellaglio *et al.* (12). Primers PEDRPO (5'-CAA GAT CGT TAA CCA GTT T-3') and PEDC1041 (5'-CCG TTG TTC CCA TAG TCT AA-3') were designed from the operon encoding pediocin PA-1 (Accession number M83924). PCR reactions were performed using a GeneAmp® PCR Instrument System 9700 (Applied Biosystems, Foster City, USA). The following conditions were used: an initial denaturation step of 94ºC for 1 min, followed by 35 cycles of 1min at 94ºC, 30sec at 50ºC and 1min at 72ºC, and final extension at 72ºC for 5min. The amplified product was visualized in a 0.8% (w/v) agarose gel stained with ethidium bromide. A band corresponding to the correct size was purified from the gel using the QIAquick PCR purification kit (QIAGEN GmbH). Purified PCR product was subject to restriction enzyme digestion with *Xho*I and *Hind*III. The digested product was visualized in a 0.8% (w/v) agarose gel stained with ethidium bromide.

PCR purified products were ligated into pGEM-T® Easy Vector (Promega, Madison, USA) and transformed into *E. coli* DH5α according to instructions of the manufacturer. Plasmids were isolated using a QIAGEN Plasmid Mini Kit and fragments sequenced on an automatic sequencer (ABI Genetic Analyzer 3130XI, Applied Biosystems) using bigdye terminator chemistry (Biosystem, Wanington, England). Sequences were analysed using DNAMAN *for Windows*® (Lynnon Biosoft, Quebec, Canada).

RESULTS AND DISCUSSION

All data represent an average of two or three repeats. The values recorded in each experiment did not vary by more than 5% and single data points are presented in the figures without standard deviation bars.

The cell-free supernatant of *L. plantarum* AMA-K inhibited the growth of *E. faecalis*, *E. mundtii*, *E. coli*, *K. pneumoniae*, *L. lactis* subsp. *lactis*, *L. casei*, *L. curvatus* and *L. sakei* (54), and *E. faecium*, *L. innocua*, *L. monocytogenes* and *L. ivanovii* subsp. *ivanovii*. According to tricine-SDS-PAGE, bacteriocin AMA-K is approximately 2.9kDa (43).

Growth of *L. plantarum* AMA-K in BHI, M17, soy milk and molasses was very similar to growth in MRS (Fig. 1). The cell density of both strains increased from OD_{600nm} 0.03 to approximately 9.8 (dilution factor taken into calculation) during 36h (Fig. 1). Low levels of bacteriocin AMA-K activity (less
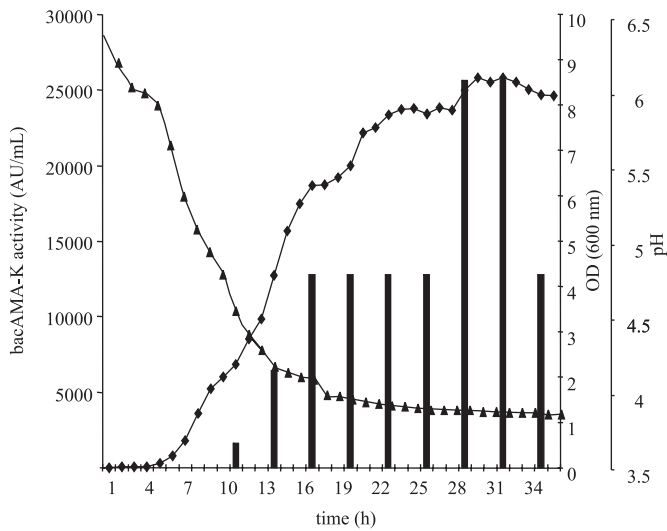
Figure 1. Growth of *L. plantarum* AMA-K (- ♦ -), production of bacteriocin AMA-K (- bars -) and changes in pH (- ▲ -) in MRS broth (Biolab).

Table 1. Influence of initial medium pH on production of bacteriocin AMA-K in MRS broth (Biolab).

	Initial pH				
	4.50	5.00	5.50	6.00	6.50
Final pH	3.45	3.52	3.60	3.60	3.60
Bacteriocin activity (AU/mL)	3200	6400	12800	12800	6400

*Compared to 12 800AU/mL (accepted as 100%) recorded in MRS broth (Biolab)

than 200AU/mL) were detected after 8h of growth in MRS broth (Fig. 1). Optimal production of bacteriocin AMA-K (25600AU/mL) was recorded after 29h (Fig. 1), and only when incubated at 30ºC or 37ºC. Bacteriocin AMA-K is a primary metabolite. Similar results have been reported for bacteriocin ST151BR (35), plantaricin Y (7) and bacteriocins produced by *P. acidilactici* (31).

The activity of bacteriocin AMA-K did not decrease during 68h of incubation at 25ºC, suggesting that extracellular proteases have not been produced. Optimal bacteriocin production (12800AU/mL) was recorded in MRS broth with an initial pH of 6.0 and 5.5 (Table 1). In MRS broth (pH 6.5) supplemented with 1mM EDTA, bacteriocin AMA-K production was 12800AU/mL, suggesting that the bacteriocin was not effected by proteases. Low levels of bacteriocin activity were recorded when the strains were cultured in MRS broth with an initial pH of 5.0 and 4.5 (3200AU/mL and 6400AU/mL, respectively, Table 1). The culture pH after 24h growth was between 3.45 and 3.60. Similar results have been reported for other bacteriocins produced by *L. plantarum* (8, 16, 37).

Growth of *L. plantarum* AMA-K in BHI broth or M17 broth adjusted to pH 6.5 yielded only 1600AU/mL of bacteriocin AMA-K (Table 2). No bacteriocin production was recorded in 10% (w/v) soy milk or 2% (w/v) molasses (Table 2). Low levels of bacteriocin AMA-K (800AU/mL) were recorded when the strains were grown in 10% (w/v) molasses (Table 2). Specific nutrients are required for the production of the bacteriocin AMA-K. This phenomenon has been observed for other bacteriocins, e.g. bacteriocins ST194BZ, ST414BZ and ST664BZ, produced by different strains of *L. plantarum* (36, 41).

Tryptone (20g/L), or a combination of tryptone and yeast extract (1:0.6), added to basal MRS medium yielded a bacteriocin level of 12800AU/mL (Table 2). Growth in the presence of a combination of tryptone and meat extract (1:0.6) reduced bacteriocin production by 50%. Growth in the presence of yeast extract (20g/L) resulted in 6400AU/mL, or in the presence of meat extract (20g/L) or a combination of meat extract and yeast extract (1:1), resulted in bacteriocin production of 3200AU/mL (Table 2).

Tryptone is the key nitrogen source needed for optimal production of bacteriocin AMA-K. Similar results have been reported for the production of plantaricin 423 (46), bacteriocin ST712BZ (40) and for bacteriocins ST151BR and ST112BR (35,38). In the case of plantaricin 423, optimal bacteriocin production was recorded in MRS broth supplemented with bacteriological peptone, followed by casamino acids, tryptone and meat extract. Stimulation of bacteriocin production by yeast extract and meat extract has been reported for helveticin J (17). As far as we could determine, this is the first indication that tryptone is the key nitrogen source needed in the production of *L. plantarum* bacteriocins.

Growth of *L. plantarum* AMA-K in the presence of glucose (20.0, and 50.0g/L) yielded 12800AU/mL of bacteriocin AMA-K (Table 2). Increased bacteriocin production (25600AU/ml) was recorded in the presence of 30g/L glucose. Lower concentrations of glucose (5.0g/L and 10.0g/L) yielded 3200AU/mL and 6400AU/mL, respectively (Table 2). Growth in the presence of maltose (20.0g/L) and sucrose (20.0g/L) yielded the same activity as 20.0g/L glucose (Table 2). Growth in the presence of mannose (20.0g/L) and fructose (20.0g/L) reduced bacteriocin production by 75%, i.e. to 3200AU/mL. In the presence of 20.0g/L lactose and 20.0g/L gluconate reduction in bacteriocin AMA-K production was even higher, i.e. 1600AU/mL. These results are surprising, since this strain was isolated from a fermented milk product, but this indicates that bacteriocin production is dependent on combination of factors. Based on these results, the production of bacteriocin AMA-K is stimulated when cells are grown in medium supplemented with 30.0g/L glucose.

Table 2. Influence of organic nitrogen, carbohydrates, growth medium and potassium on the production of bacteriocin AMA-K.

Component	Concentration(g/L)	pH	Bacteriocin activity(AU/mL)
Tryptone	20.0	3.84	12800
Meat extract	20.0	3.78	3200
Yeast extract	20.0	3.84	6400
Tryptone + meat extract	12.5 + 7.5	3.83	6400
Tryptone + yeast extract	12.5 + 7.5	3.71	12800
Meat extract + yeast extract	10.0 + 10.0	3.76	3200
Tryptone + meat extract + yeast extract	10.0 + 5.0 + 5.0	3.54	12800
Glucose	5.0	3.64	3200
-"-	10.0	3.59	6400
-"-	20.0	3.54	12800
-"-	30.0	3.52	25600
-"-	50.0	3.50	12800
Fructose	20.0	3.73	3200
Sucrose	20.0	3.66	12800
Maltose	20.0	3.63	12800
Mannose	20.0	3.62	3200
Lactose	20.0	3.53	1600
Gluconate	20.0	5.23	1600
Glycerol	0	3.54	12800
-"-	1.0	3.66	12800
-"-	5.0	3.67	6400
-"-	10.0	3.70	1600
-"-	20.0	3.68	800
-"-	50.0	3.68	200
MRS	50.0	3.54	12800
BHI	37.0	5.79	1600
M17	42.5	559	1600
Soy flour	100.0	4.47	0
Molasses	100.0	4.33	800
-"-	20.0	3.89	0
$K_2HPO_4 + KH_2PO_4$	2.0 + 2.0	3.70	6400
K_2HPO_4	2.0	3.54	12800
-"-	5.0	3.73	3200
-"-	10.0	3.93	3200
-"-	20.0	4.38	400
KH_2PO_4	2.0	3.66	6400
-"-	5.0	3.82	1600
-"-	10.0	3.69	400
-"-	20.0	3.75	0
Magnesium sulphate	0	3.48	1600
Manganese sulphate	0	3.80	3200
Tri-ammonium citrate	0	3.63	3200
-"-	2.0	3.54	12800
-"-	5.0	3.54	12800
-"-	10	4.04	3200
Tween 80	0	3.60	3200
-"-	0.5	3.59	6400
-"-	1.0	3.58	12800
-"-	1.5	3.88	12800
-"-	2.0	3.86	12800

	(mg/mL)		
Cyanocobalamin (Vit. B$_{12}$)	1.0	3.74	3200
Thiamine (Vit. B$_1$)	1.0	3.65	6400
DL-6,8-thioctic acid	1.0	3.66	3200
L-ascorbic acid (Vit. C)	1.0	3.66	6400
Control	0	3.54	12800

*Compared to 12 800 AU/mL (accepted as 100%) recorded in MRS broth (Biolab)

Bacteriocin AMA-K production was affected differently by the presence of different concentrations of K$_2$HPO$_4$ or KH$_2$PO$_4$. Optimal bacteriocin AMA-K production (12800AU/mL) was recorded in the presence of 2.0g/L K$_2$HPO$_4$. Higher concentrations K$_2$HPO$_4$ had a negative effect on bacteriocin AMA-K production (Table 2). However, replacing K$_2$HPO$_4$ with KH$_2$PO$_4$ resulted in reduction of bacteriocin AMA-K production (Table 2). A combination of K$_2$HPO$_4$ and KH$_2$PO$_4$ (2.0g/L and 2.0g/L) resulted in the reduction of bacteriocin AMA-K by 50% (Table 2). In the case of plantaricin UG1, 7.0g/L K$_2$HPO$_4$ resulted in increased activity (13). Different concentrations of K$_2$HPO$_4$ and KH$_2$PO$_4$ did not significantly affect production of bacteriocin ST712BZ, produced by *L. pentosus* ST712BZ (40). In the case of bacteriocin ST112BR, higher levels of activity was recorded when the medium contained 5.0g/L, 10.0g/L and 20.0g/L KH$_2$PO$_4$ (23). The optimal concentration of K$_2$HPO$_4$ required for plantaricin ST31 production was between 2.0g/L and 5.0g/L (37).

Production of bacteriocin AMA-K was the highest (12800AU/mL) in the absence or in presence of very low (1.0g/L) concentration of glycerol (Table 2). Glycerol concentrations of 5.0g/L and higher (up to 50.0g/L) led to progressively decreased levels of bacteriocin AMA-K production (Table 2). Similar results were reported for the production of bacteriocins ST151BR, ST112BR, ST712BZ and plantaricin ST31 (35,37, 38,40). An increase in glycerol leads to a lowering in water activity. The production of bacteriocin AMA-K may be influenced by osmotic stress or by binding of bacteriocin to the cell membranes or other molecules, initiated by presence of the glycerol.

Optimal bacteriocin AMA-K production was recorded in presence of 1.0g/L, 1.5g/L and 2.0g/L Tween 80. Lower concentrations of Tween 80 have a negative effect on bacteriocin AMA-K production (Table 2).

Production of bacteriocin AMA-K requires the presence of magnesium sulphate and manganese sulphate as a part of the production medium. Exclusion of these salts resulted in reduction of bacteriocin AMA-K production (Table 2). Similar results were recorded for the effect of tri-ammonium citrate. Normally this component is present in MRS medium at a level of 2.0g/L. At this concentration optimal bacteriocin AMA-K production was recorded. Exclusion of tri-ammnium citrate from the media formula

resulted in reduction of bacteriocin activity to 3200AU/mL. However, increasing the concentration to 10.0g/L had a similar effect on bacteriocin AMA-K production (3200AU/ml, Table 2).

Reduction in bacteriocin AMA-K production to 6400AU/mL was recorded in the presence of Vit. B$_1$ or DL-6,8-thioctic acid (Table 2). However, a decrease to 3200AU/ml was recorded then MRS were supplemented with Vit. B$_{12}$ or Vit. C (Table 2). In the case of bateriocin ST194BZ, produced by *L. plantarum* ST194BZ, vitamins Vit. B$_{12}$ and Vit. B$_1$ had no effect on bacteriocin production, but addition of Vit. C or DL-6,8-thioctic acid had a negative effect, reducing the bacteriocin ST194BZ production with 50% (36).

The addition of 25 600 AU/ml bacteriocin AMA-K to a 3-h-old culture of *L. innocua* LMG13568, *L. ivanovii* subsp. *ivanovii* ATCC19119 and *L. monocytogenes* ScottA (OD$_{600nm}$ = 0.1) resulted in growth inhibition for 9h (Fig. 2), suggesting that the mode of activity of bacteriocin AMA-K is bacteriocidal. Addition of the same concentration of bacteriocin AMA-K to stationary-phase cells of *L. innocua* LMG13568, *L. ivanovii* subsp. *ivanovii* ATCC19119 and *L. monocytogenes* ScottA, resulted in no significant growth inhibition (results not shown). This suggested that bacteriocin AMA-K is only active against actively growing cells.

Bacteriocin AMA-K was adsorbed at 75% to cells of *L. innocua* LMG13568, *L. monocytogenes* ScottA and *L. ivanovii* subsp. *ivanovii* ATCC19119. (Table 3). Different levels of adsorption was observed in our previous study for bacHV219, but in general highest levels were observed in adsorption to sensitive strains compared to strains resistant to the effect of bacteriocin (42). Similar results have been reported for pediocin N5p (24), *viz.* 100% adsorption to sensitive cells of *O. oeni* X2L, 80% to *Lactobacillus hilgardii* and *O. oeni* L10, and 70% to *L. hilgardii* 6D (24). Adsorption of pediocin N5p to resistant bacteria was below 20% (24). Buhnericin LB adsorbed 100% to sensitive cells of *L. plantarum*, *Pediococcus dextranicus*, *O. oeni* and *E. faecalis*, but also 100% to an insensitive strain of *Pediococcus cerevisiae* (47). In the case of plantaricin 423, adsorption ranged from 17% for *Streptococcus caprinus* ATCC 700066 to 67% for *L. plantarum* LMG 13556, *L. curvatus* DF38, *L. innocua* LMG 13568 and *L. sakei* DSM 20017 (39). Strains sensitive to plantaricin 423 adsorbed the peptide stronger (39).
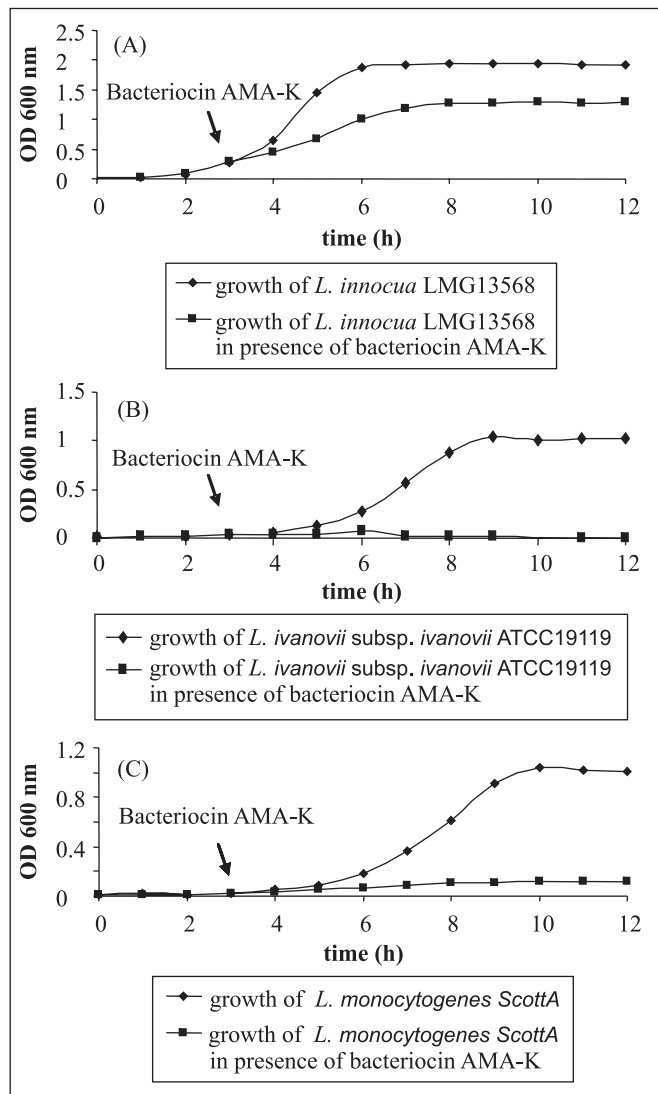
Figure 2. Effect of bacteriocin AMA-K on (A) *L. innocua* LMG13568, (B) *L. ivanovii* susbp. *ivanovii* ATCC19119 and (C) *L. monocytogenes* ScottA. (-◆-) represent the growth of *L. innocua* LMG13568, *L. monocytogenes* ScottA and *L. ivanovii* subsp. *ivanovii* ATCC19119 without added bacteriocins (controls) and (-□-) growth with added bacteriocin AMA-K after 3h. The arrow indicates the point at which the bacteriocins were added.

Optimal adsorption of bacteriocin AMA-K (75%) to *L. innocua* LMG13568, *L. monocytogenes* ScottA and *L. ivanovii* subsp. *ivanovii* ATCC19119 were recorded at pH 7.0. lower levels of pH (3.5) resulted in reduction of the adsorption of bacteriocin AMA-K to this *Listeria* species to 50%. However, in an experiment at pH 5.5, reduction to 50% were recorded for adsorption to *L. innocua* LMG13568 and to *L. monocytogenes*

ScottA, but no change in level of adsorption was recorded for *L. ivanovii* susbp. *ivanovii* ATCC19119 (Table 3). These results show the potential of the application of this bacteriocin at neutral or moderate acid pH. Differences in adsorption affected by pH rates may be due to specific interaction between bacteriocin AMA-K and the target strain. In the case of buchnericin LB, optimal adsorption to *L. plantarum* was recorded at pH 5.0 – 8.0 (47). Optimal adsorption of plantaricin 423 to *E. faecium* HKLHS was recorded between pH 8.0 and 10.0, and to *L. sakei* DSM20017 between pH 2.0 and 6.0 (39).

Temperature has effect on bacteriocin AMA-K adsorption to *L. innocua* LMG13568, *L. monocytogenes* ScottA and *L. ivanovii* susbp. *ivanovii* ATCC19119. At 30ºC and 37ºC levels of 75% adsorption were observed for all tested *Listeira* species (Table 3). At 45ºC increased adsorption of bacteriocin AMA-K was recorded to cells of *L. ivanovii* susbp. *ivanovii* ATCC19119 (87.5%) and to *L. monocytogenes* ScottA (100%) (Table 3). However, temperature of 4ºC and 15ºC resulted in reduction to 50% of adsorption of bacteriocin AMA-K to cells of *L. innocua* LMG13568 and *L. ivanovii* subsp. *ivanovii* ATCC19119 (Table 3). It was previously reported for bacHV219 that an increase in temperature from 25ºC to 60ºC had a negative effect on the adsorption of bacHV219 to *E. faecium* HKLHS. Forty percent adsorption was recorded between 25ºC and 60ºC, with optimum adsorption (80%) at 4ºC. Complete adsorption (100%) to *E. faecalis* E88 was recorded after treatment at 4ºC, 10ºC, 45ºC and 60ºC. A 20% decrease in adsorption was recorded at 37ºC (42).

Adsorption was affected by temperature. Similar results were observed for other bacteriocins. In the case of buchnericin LB, identical adsorption levels to cells of *L. plantarum* was recorded after treatment at 0, 10, 25, 50 and 80ºC (47). Changes in temperature had no effect on the adsorption of plantaricin 423 to *E. faecium* HKLHS (50).

Decreased adsorption of bacteriocin AMA-K to *L. innocua* LMG13568, *L. monocytogenes* ScottA and *L. ivanovii* susbp. *ivanovii* ATCC19119 was observed in the presence of Tween 20, Tween 80 and different concentrations of NaCl (Table 3). Ascorbic acid and potassium sorbate not effect adsorption of bacteriocin AMA-K to cells of *L. innocua* LMG1568, but reduced this process to cells of *L. monocytogenes* Scott and to *L. ivanovii* subsp. *ivanovii* ATCC19119 (Table 3). The presence of 1% sodium nitrate increased adsorption of bacteriocin AMA-K to cells of *L. innocua* LMG13568 and *L. monocytogenes* ScottA (Table 3). Increased adsorption of bacHV219 to *E. faecium* HKLHS was detected in the presence of Triton X-100, β-mercapto-ethanol, chloroform, NaCl, KH_2PO_4 and $MgCl_2$ (42). Adsorption of bacHV219 to *E. faecalis* E88 increased in the presence of Na-acetate, Na_2CO_3, Triton X-100, 80% ethanol, methanol, K_2HPO_4, KH_2PO_4, $MgCl_2$, KCl, Tris and NH_4-citrate (42).

An increase in the adsorption of plantaricin 423 to *E. faecium* HKLHS was observed in the presence of Triton X-100, Triton

X-114 and chloroform (39). Adsorption of bacHV219 to *E. faecalis* E88 increased in the presence of Na-acetate, Na_2CO_3, Triton X-100, 80% ethanol, methanol, K_2HPO_4, KH_2PO_4, $MgCl_2$, KCl, Tris and NH_4-citrate. *L. sakei* DSM 20017 treated with NaCl, K_2HPO_4, KH_2PO_4, $MgCl_2$, KCl, KI, Tris, NH4-citrate, Na_2CO_3, SDS, β-mercapto-ethanol, 80% ethanol and methanol led to a reduction in the adsorption of plantaricin 423 (39). No change in adsorption was observed in the presence of Na-acetate or EDTA, whereas an increase in adsorption was observed in the presence of Triton X-100, Triton X-114 and chloroform (39). Adsorption of buchnericin LB to *L. plantarum* was reduced by NaCl, NH_4Cl, $MgCl_2$, KCl, KI and Tris. Treatment of cells with NH_4-citrate, Na-acetate, $NaCO_3$, EDTA SDS, triton-X, 2-mercapto-ethanol, 80% ethanol and 80% methanol had no effect on adsorption of buchnericin LB to *L. plantarum* (47). Adsorption of pediocin N5p to *P. pentosaceus* E5p increased in the presence of $MgCl_2$, $MgSO_4$, $MnCl_2$, $MnSO_4$, whereas NaCl, KCl, KI, NH_4Cl, $CaCl_2$, Na_3PO_4, Na_2SO_4, EDTA and ethanol had no affect on adsorption (31). Organic salts and Na-acetate reduced pediocin N5p adsorption to target cells. Adsorption of pediocin N5p increased with 25% in the presence of SDS (24).

L. plantarum AMA-K has a 1044bp fragment corresponding to that recorded for pediocin PA-1 (Fig. 3A). Digestion with *Xho*I and *Hin*dIII showed that the purified PCR product differed from that obtained for *L. pla*ntarum 423 (Fig. 3B). The PCR product from *L. plantarum* 423 was digested to 2 fragments by *Xho*I. However, the PCR product from *L. plantarum* AMA-K DNA was digested to 2 products only by *Hin*dIII. The difference in digested profiles were expected based on the genetic sequence of the genes of plantaricin 423 and pediocin PA-1 (45). The sequences of the PCR product using DNA from *L. plantarum* AMA-K was identical to that reported for pediocin PA-1 (25). Pediocin PA-1 biosynthesis involves a DNA fragment of approximately 3.5 kb, comprising the four genes *pedA*, *pedB*, *pedC,* and *pedD* (25). This results show that bacteriocin AMA-K share high homology to pediocin PA-1.

CONCLUSIONS

Bacteriocin AMA-K inhibits the growth of *E. faecalis*, *E. mundtii*, *E. coli*, *K. pneumoniae*, *L. lactis* subsp. *lactis*, *L. casei*, *L. curvatus*, *L. sakei*, *E. faecium*, *L. innocua*, *L. monocytogenes* and *L. ivanovii* subsp. *ivanovii*. Growth of strain AMA-K in BHI, M17, soy milk and molasses was similar to growth in MRS.

Table 3. Effect of temperature, pH and chemicals on adsorption of bacteriocin AMA-K to *L. innocua* LMG13568, *L. ivanovii* susp. *ivanovii* ATCC19119 and *L. monocytogenes* ScottA.

	L. innocua LMG 13568	*L. ivanovii* susbp. *ivanovii* ATCC 19119	*L. monocytogenes* ScottA
Effect of temperatures (ºC):			
4	50	50	75
15	50	50	75
30	75	75	75
37	75	75	75
45	75	87.5	100
pH			
3.5	50	50	50
5.5	50	75	50
7.0	75	75	75
Chemicals (1%)			
Tween 80	25	25	25
Tween 20	25	25	50
Ascorbic acid	75	50	50
Potassium sorbate	75	50	50
Sodium nitrate	87.5	75	87.5
NaCl (0.5%, 1.0%, 1.5% and 2.0%)	50	50	50
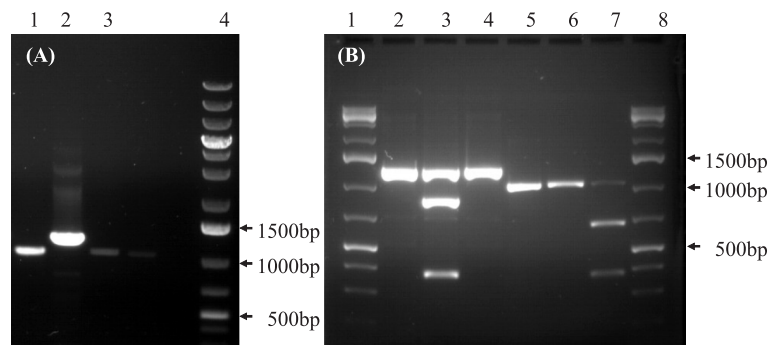
Figure 3. PCR plantaricin and restriction
GEL (A): DNA banding patterns obtained after PCR with primers to PA-1 genes. Lane 1: Strain *L. plantarum* AMA-K (10 µL PCR product loaded), lane 2: Strain *L. plantarum* 423 (10 µL PCR product loaded), lane 3: Strain *L. plantarum* AMA-K (3 µL PCR product loaded), Line 4: O'GeneRuler™ 1kb DNA Ladder (Fermentas).
GEL (B): Restriction enzyme analysis using *Xho*I and *Hin*dIII. Lines 1 and 8: O'GeneRuler™ 1kb DNA Ladder (Fermentas); line 2: plantaricin 423-uncut; line 3: plantaricin 423-*Xho*I; line 4: plantaricin 423-*Hin*dIII; line 5: bacteriocin AMA-K-uncut; line 6: bacteriocin AMA-K-*Xho*I; line 7: bacteriocin AMA-K-*Hin*dIII.

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Optimal bacteriocin production was recorded in MRS broth with an initial pH of 6.0 and 5.5. After 20h of incubation in all media compositions tested, MRS supplemented with tryptone (20.0g/L), tryptone and yeast extract (12.5g/L and 7.5g/L), glucose (20.0 and 50.0g/L), sucrose (20.0g/L), maltose (20g/L), glycerol (up to 1.0g/L), K_2HPO_4 (2.0 g/L), tri-ammonium citrate (5.0g/L) yielded 12800AU/mL. Glucose at 30g/L increased bacteriocin AMA-K production by 100%. Optimal adsorption of bacteriocin AMA-K (75%) to *Listeria* strains was recorded at pH 7.0. However, temperatures of 4°C and 15°C resulted in reduction of 50% of adsorption of bacteriocin AMA-K to cells of *L. innocua* LMG13568 and *L. ivanovii* subsp. *ivanovii* ATCC19119. Decreased adsorption of bacteriocin AMA-K to *Listria* strains was observed in the presence of Tween 20, Tween 80 and different concentrations of NaCl. Ascorbic acid and potassium sorbate did not effect adsorption of bacteriocin AMA-K to cells of *L. innocua* LMG1568, but reduced this process in cells of *L. monocytogenes* Scott and to *L. ivanovii* subsp. *ivanovii* ATCC19119. The presence of 1% sodium nitrate increased adsorption of bacteriocin AMA-K to cells of *L. innocua* LMG13568 and *L. monocytogenes* ScottA. Bacteriocin AMA-K shares high homology to pediocin PA-1.

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RESUMO

Otimização da produção de bacteriocina por *Lactobacillus plantarum* AMA-K isolado de Amasi, um produto lácteo fermentado de Zimbabwe, e estudo da adsorção da bacteriocina AMA-K à *Listeria innocua*, *Listeria monocytogenes* e *Listeria ivanovii* subsp.*ivanovii*

A bacteriocina AMA-K produzida por *Lactobacillus plantarum* AMA-K inibe a multiplicação de *Enterococcus* spp, *Escherichia coli*, *Klebsiella pneumoniae* e *Listeria* spp. A multiplicação da cepa AMA-K em BHI, leite de soja e melaço foi semelhante à multiplicação em MRS. O efeito de fontes de nitrogênio orgânico, carboidratos, glicerol, K_2HPO_4 e KH_2PO_4, $MgSO_4$, $MnSO_4$, citrato de triamônio, Tween 80, vitaminas e pH inicial sobre a bacteriocina AMA-K foi determinada. O modo de ação da bacteriocina AMA-K foi estudado. O efeito da bacteriocina AMA-K sobre *Listeria innocua* LMG13568, *Listeria ivanovii* subsp.*ivanovii ATCC19119* e *Listeria monocytogenes* Scott A foi determinado. A adsorção da bacteriocina AMA-K às células-alvo em diferentes temperaturas, pH e na presença de Tween 20, Tween 80, ácido ascórbico, sorbato de potássio, nitrato de sódio a cloreto de sódio foi avaliada. A bacteriocina AMA-K apresenta grande homologia a pediocina PA-1.

Palavras-chave: bacteriocina AMA-K, *Lactobacillus plantarum,* amasi

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