



Characterization and Identification of Some Bacteriocins Produced by Lactic Acid Bacteria Isolated from Fermented Food Products

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ABSTRACT

Lactic acid bacteria (LAB) such as *Pediococcus halophilus* FCF7 and *Leuconostoc gelidum* FALB 1 were isolated from fermented corn flour and African locust bean using pour plate technique and were propagated in De Man Rogosa Sharpe (MRS) broth separately for bacteriocins production. Bacteriocins produced by *Pediococcus halophilus* FCF7 and *Leuconostoc gelidum* FALB 1 were purified, characterized and identified. Variations in total activity, specific activity, protein contents and bacteriocin activity during the process of bacteriocin purification were observed. *L. gelidum* FALB 1 had higher bacteriocin recovery of 19.0% while *P. halophilus* FCF7 had 13.68% bacteriocin recovery. The bacteriocins produced by *L. gelidum* FALB1 inhibited all test isolates, while bacteriocins produced by *P. halophilus* FCF7 inhibited all test isolates except *Listeria monocytogenes* W6, *Shigella dysenteriae* N11 and *Klebsiella ozaenae* W24. Bacteriocins produced by *P. halophilus* FCF7 and *L. gelidum* FALB1 exhibited bacteriocin activity of 8000 and 5800 respectively. The bacteriocins were stable, slightly stable and unstable at various exposed, storage temperatures and at different pH levels. The bacteriocins produced by *P. halophilus* FCF7 and *L. gelidum* FALB1 were identified as pediocin and leucocin respectively. Bacteriocins from the generally regarded as safe (GRAS) - LAB, have received significant attention as a novel approach to the control of pathogens in foods and having the ability to prolong the shelf life of fermented foods.

Keywords: Characterization, pediocin, leucocin, purification, MRS broth, LAB

INTRODUCTION

Lactic acid bacteria are described as microaerophilic as they do not utilize oxygen. All lactic acid producers are non-motile Gram-positive rods that need complex carbohydrate substrates as sources of energy. Most LAB are aerotolerant anaerobes which lack cytochromes and porphyrins and therefore are catalase and oxidase negative (Adams and Moss, 2008). Fermentation of various foods by LAB is one of the oldest forms of Biopreservation practiced by mankind. Bacterial antagonism had been recognized for over a century but in recent years this phenomenon has received more scientific attention, particularly in the use of various strains of lactic acid bacteria. One important attribute of LAB is their ability to produce antimicrobial compounds called bacteriocin. In recent years, interest in the compound has grown substantially due to their potential usefulness as natural substitute for chemical food preservatives in the production of foods with enhanced shelf life and/or safety. This balance is achieved by their inhibitory effects upon the

harmful pathogenic microorganisms (Savadogo *et al.*, 2006). The fermentation processes increase the shelf-life of the products as well as add to the taste and improves the digestibility of milk. Historically, lactic acid bacteria from the genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Pediococcus* are the main species involved. Several more have been identified but with minor significance or role in lactic acid fermentations (Food and Agriculture Organization, FAO, 2013).

Bacteriocins are naturally occurring antibiotic peptides produced by Gram positive bacteria, and may contain as much as 24 amino acids. Some Bacteriocins are lantibiotics, which means that they are post translationally modified so as

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to encompass the amino acid lanthionine or “Lan” (Chatterjee *et al.*, 2005). Bacteriocins, the antimicrobial substances of LAB have gained tremendous attention as potential bio preservatives. Bacteriocins are ribosomally synthesized, extracellularly released bioactive peptides or peptide complexes, having a bactericidal or bacteriostatic activity. They differ from most therapeutic antibiotics in being proteinaceous agents that cannot potentially elicit allergic reactions in humans and other medical problems (Amani, 2012). Soomro *et al.* (2002) and Kacem *et al.* (2005) had characterized and identified bacteriocins such as Nisin, Pediocin A, Pediocin AcH, Lecucocin, Helveticin J and Camobacteriocin produced by LAB isolated from different food sources. This study is aimed at characterization and identification of some bacteriocins produced by lactic acid bacteria isolated from fermented corn flour (*Akamu*) and fermented African locust bean (*Daddawa*).

METHODOLOGY

Collection of samples

Fermented corn flour (*Akamu*), Fermented African locust bean (*daddawa*), Nono and Wara were collected separately in clean sample bottles from Bosso Market, Minna, Niger State, Nigeria and were transferred to the Laboratory for the Isolation of Lactic acid bacteria (LAB) and test microorganisms from milk products.

Isolation of lactic acid bacteria (LAB) from fermented foods

Twenty-five grams (25g) each of fermented corn flour and fermented African locust bean were collected and transferred into sterile sample bottles and 225ml of buffered peptone water, Bpw (Oxoid) was added to obtain 1:10 dilution for each sample respectively. The samples were blended for 1 minute respectively. Serial dilutions of the samples were done in 0.1% peptone water. Serially diluted samples were plated on lactic acid bacteria medium (LABM) and incubated ‘anaerobically’ at 37°C for 24 hours. Colonies that appeared on the plates were counted using the colony counter and the results recorded as colony forming units per gram (cfu/g). Pure cultures were obtained by repeated sub-culturing of the isolates on fresh medium. Pure cultures were maintained on agar slants for further characterization and identification (Mohammed, 2015).

Isolation of microorganisms from milk products

Streak method of isolation described by Cheesbrough. (2003) and Bromberg (2004) were used. microorganisms were isolated from nono and wara. Sterile wire loop (heat to red hot, then allowed to cool) was used to pick samples from the blended wara samples. The samples were streaked on nutrient agar and incubated at 37°C for 24 hours. For the nono samples, sterile wire loop was dipped into the serially diluted samples and streaked on nutrient agar for each sample respectively and incubated at 37°C for 24 hours. The colonies that appeared on the medium were maintained on agar slants for further characterization and identification.

Characterization of and identification of microbial isolates

Microbial isolates were characterized and identified based on colony morphology, cell morphology and biochemical tests described by Cheesbrough. (2003); Oyeleke and Manga (2008). The LAB were identified as *Pediococcus halophilus* FCF7 and *Leuconostoc gelidum* FALB using the scheme of Begey’s manual of determinative bacteriology (1984).

Bacteriocin Production

Lactic acid bacteria were propagated in 1000 ml of De Man Rogosa Sharpe (MRS) broth (pH5.8) and incubated at 30°C for 48 hours (Kacem *et al.*, 2005).

Extraction of cell free bacteriocins

For extraction of bacteriocins, a cell free solution was obtained by centrifugation (10,000 rpm for 20 minutes). The culture was then adjusted to pH 7.0 using 1M NaOH to exclude the antimicrobial effect of organic acid, then followed by filtration of the supernatant through 0.2µm pore size cellulose acetate filter to obtain crude bacteriocin (Kacem *et al.*, 2005).

Elimination of inhibitory activity

Inhibition activity from hydrogen peroxide (H₂O₂) was eliminated by the addition of 5 mg/ml catalase as described by Kacem *et al.* (2005).

Testing of bacteriocin broth culture

The broth culture was tested for pH, bacteriocin activity and concentration of bacteriocin samples as described by Ogunbanwo *et al.* (2003).

Purification of Bacteriocin Samples

The bacteriocins were purified. The methods of Kacem *et al.* (2005) was used. Three steps were involved as described below:

i. Ammonium sulphate precipitation

The crude bacteriocin samples produced were treated with solid ammonium sulphate (Mallinckrodt Chemical, Inc. Paris, France) to 10%, 20% 30%, 40% and 50% saturation to salt out. The mixture was stored at 4°C for 2 hours and then centrifuged at 10,000 rpm for 1 hour. The precipitate was re-suspended in 25 ml of 0.5 M potassium phosphate buffer (pH 7.0). Then the suspension was dialysed in tabular cellulose membrane against 2 litres of the buffer for 18 hours in dialysis tubing. The products of ammonium sulphate precipitation constituted Fraction I.

ii. Trichloroacetic acid (TCA) precipitation

Five percent (5%) equivalent of TCA (5ml) was added to 25 ml of fraction 1 to precipitate the protein (bacteriocin). The mixture was centrifuged at 10,000 rpm for 10 minutes after which the supernatant was decanted. The resulting pellet was dissolved in 2ml of potassium phosphate buffer to obtain Fraction II.

iii. Ultrafiltration Analysis

The fraction II (Trichloroacetic acid precipitation) bacteriocin sample was re-suspended to $\frac{1}{3}$ volume in potassium phosphate buffer (50mM, pH 7.0). Several aliquots (1ml) were ultrafiltered through various filtron membranes (Filtron Technology Corp; Northborough, Massachusetts), including 100,000, 10,000, 1,000 Kilogram per Dalton molecular sizes. Bacteriocin activity was determined and protein concentration of the fractions were determined by the Bradford method (Bradford, 1976).

Characterization of Bacteriocins

The purified bacteriocin samples were characterized with respect to thermal stability, pH stability, stability during storage and other properties exhibited by bacteriocins (Soomro *et al.*, 2002; Ogunbanwo *et al.*, 2003). These are described below:

Temperature Stability of Bacteriocins:

The purified bacteriocin samples (400µl) were dispensed into test tubes (13mm x100mm) using pipette (micropipette) and were exposed to the following oven temperatures: 40, 60, 80, 100 and 121°C. Aliquots of each fraction was removed after 30 minutes and tested for bacteriocin activity on the test isolates (*Escherichia coli* N2, *Pseudomonas aeruginosa* N7, *Listeria monocytogenes* W6, *Shigella dysenteriae* N11, *Bacillus cereus* W18, *Salmonella typhimurium* N8, *Escherichia coli* W4, *Proteus vulgaris* W7, *Klebsiella aerogenes* N12, *Staphylococcus aureus* N16b, *Bacillus subtilis* N20, *Klebsiella ozaenae* W24, *Proteus mirabilis* N16a).

pH stability of Bacteriocins:

The purified bacteriocin (400µl) were dispensed into different test tubes (13mm x100mm) using pipette (micropipette) and bacteriocin adjusted to pH 2, 4, 6, 8, 10, 12 with 0.1 M sodium hydroxide (NaOH) and incubated at room temperature for 24 hours (Kacem *et al.*, 2005). The bacteriocin was assayed against the test isolates (*Escherichia coli* N2, *Pseudomonas aeruginosa* N7, *Listeria monocytogenes* W6, *Shigella dysenteriae* N11, *Bacillus cereus* W18, *Salmonella typhimurium* N8, *Escherichia coli* W4, *Proteus vulgaris* W7, *Klebsiella aerogenes* N12, *Staphylococcus aureus* N16b, *Bacillus subtilis* N20, *Klebsiella ozaenae* W24, *Proteus mirabilis* N16a).

Stability of Bacteriocins during Storage at Different Temperature:

Purified bacteriocins were kept in the test tubes (13mm x100mm) and stored at 4°C and 10°C (refrigeration temperature) and 24±1°C (room temperature) for 30 minutes. Samples were taken from the stored bacteriocin to determine the bacteriocin activity (Kacem *et al.*, 2005).

Other Properties of Bacteriocins:

The purified bacteriocin properties were based on spectrum (broad, narrow) after diameter of zones of inhibition measurement. Bactericidal (killing), bacteriostatic (inhibiting) characteristics. The test plates were again incubated for another 48 hours and were observed for growth (growth-indicate bacteriostatic (inhibition), while no growth-indicate bactericidal (kill). Bacteriocin produced early or late in the cycle were determined when bacteriocins were tested against the test isolates for bacteriocin activity (AU/mL) every 12 hours.

Class of bacteriocins were determined using bacteriocin characteristics (Soomro *et al.*, 2002; Mohammed *et al.*, 2012).

Antibacterial Activity of Bacteriocins

Agar well procedures of Kacem *et al.* (2005) were used. Aliquots of 500 μ l from each bacteriocin was placed in agar wells (the wells were bored using 5 mm cork borer) in Petri dishes seeded with the bioassay strains (*Escherichia coli* N2, *Pseudomonas aeruginosa* N7, *Listeria monocytogenes* W6, *Shigella dysenteriae* N11, *Bacillus cereus* W18, *Salmonella typhimurium* N8, *Escherichia coli* W4, *Proteus vulgaris* W7, *Klebsiella aerogenes* N12, *Staphylococcus aureus* N16b, *Bacillus subtilis* N20, *Klebsiella ozaenae* W24, *Proteus mirabilis* N16a) and incubated overnight at 37°C and the diameters of the zone of inhibition was measured. The antimicrobial activity of the bacteriocins produced was defined as the reciprocal of the highest dilution showing inhibition of microorganisms multiplied by 100 and it is expressed as activity units per ml (AU/mL) (Ogunbanwo *et al.*, 2003).

Results

The fermented corn flour and fermented African locust bean analyzed had different species of lactic acid bacteria (LAB) in them. *Pediococcus halophilus* FCF 7 and *Leuconostoc gelidum* FALB 1 was isolated and identified to species level (Table 1). The *Pediococcus halophilus* FCF 7 and *Leuconostoc gelidum* FALB 1 were selected after vigorous screening based on its ability to grow in De Man Rogosa Sharpe broth to produce, also through spectrophotometric analysis at 580 nm wave length, pH, bacteriocin activity (AU/mL). It was observed that *Pediococcus halophilus* FCF 7 had optical density of 0.80, at pH of 4.00 and bacteriocin activity of 5800 AU/mL, while *Leuconostoc gelidum* FALB 17 had optical density of 0.90, at pH of 4.90 and bacteriocin activity of 8000 AU/mL (Table 2). *Pediococcus halophilus* FCF7 exhibited bacteriocin activity of 6000

AU/mL, while 8200 AU/mL bacteriocin activity was exhibited by *Leuconostoc gelidum* FALB1 after the purification processes. It was observed that there were variations in bacteriocin activity, total activity, specific activity and protein content of bacteriocin produced by *Pediococcus halophilus* FCF 7 and *Leuconostoc gelidum* FALB 1. It was also observed that the specific activity, amount of protein and recovery of bacteriocins was decreasing after each treatment process of bacteriocins (Table 3). *Leuconostoc gelidum* FALB1 had the highest bacteriocin recovery (19.00%) while *Pediococcus halophilus* FCF 7 had 13.68% bacteriocin recovery at the end of the purification process (Table 3). Bacteriocin produced by *Pediococcus halophilus* FCF 7 and *Leuconostoc gelidum* FALB 1 were most heat stable at 40°C and 60°C for 30 minutes, slightly stable at 80°C and 100°C but unstable at 121°C for 30 minutes (Table 4). The bacteriocin produced by *Pediococcus halophilus* FCF 7 and *Leuconostoc gelidum* FALB 1 maintained full stability after storage for 60 days at 4°C, slightly stable at 10°C and unstable at 24 \pm 1°C. It was also observed that the bacteriocin produced by *Pediococcus halophilus* FCF 7 and *Leuconostoc gelidum* FALB 1 were mostly stable at pH 2,4 and 6, slightly stable at pH 8 and 10 unstable at pH 12. The bacteriocins also exhibited some properties that justified their characterization. These properties include broad and narrow spectrum inhibition of pathogenic and spoilage microorganisms, bactericidal or bacteriostatic effects and the time of bacteriocin production by test isolates. Bacteriocins from the LAB had broad spectrum of inhibition against the test isolates. The *Leuconostoc gelidum* FALB 1 produced bacteriocin early in the cycle while *Pediococcus halophilus* FCF 7 produced bacteriocin late in the cycle (Table 4). The bacteriocins produced by *L.gelidum* FALB1 inhibited all the test isolates while bacteriocins produced by *P.halophilus* FCF7 inhibited all the test isolates except *Listeria monocytogenes* W6, *Shigella dysenteriae* N11 and *Klebsiella ozaenae* W24 (Table 5).

Table 1 .Cultural and Biochemical Characteristics of bacteriocin producing lactic acid bacteria isolated from Fermented food products

Isolates codes	Colony Morphology	Cell morphology	Gram staining	Oxidation test	Mannitol activity	Catalase	NH3 activity from arginine	Gelatine liquefaction	Sugar Fermentation				Proable Organisms	
									Glucose	Sucrose	Fructose	Lactose		
FCF 7	Grey white	Cocci in two's	G+	-	-	-	+	-	A	AG	AG	AG	AG	<i>Pediococcus halophilus</i> FCF 7
FALB1	Irregular cocci	Cocci	G+	-	-	-	+	-	A	AG	AG	A		<i>Leuconostoc gelidum</i> FALB1

FCF : fermented corn flour, FALB: fermented African locust bean,+ : positive result,-: Negative result, A : Acid production ,G :Gas production, AG :Acid and Gas production, G+ = Gram positive

Table 2. Production of bacteriocin by *Pediococcus halophilus* and *Leuconostoc gelidum*

Isolates	optical density of LAB (580nm)	pH of bacteriocins	Bacteriocin activity (Au/mL)
<i>Pediococcus halophilus</i> FCF7	0.8	4.00	5800
<i>Leuconostoc gelidum</i> FALB1	0.9	4.90	8000

FCF: FCF : fermented corn flour, FALB: fermented African locust bean ,Au/ml: Activity unit per milliliter, nm: nanometer

Table 3. Purification of bacteriocins produced by *Pediococcus halophilus* FCF7 and *Leuconostoc gelidum* FALB1

Isolates	Purification stages	Volume of culture supernatant (ml)	Activity of bacteriocins (Au/ml)	^I Total activity	^{II} protein (µg/ml)	^{III} specific activity	^{IV} Recovery (%)
<i>Pediococcus halophilus</i> FCF7	Culture supernatant	1000	5800	5800000	380	15.26	100
	Ammonium sulphate Precipitation	50	5800	2920000	200	29.00	52.62
	Trichloroacetic acid Precipitation	2.5	6000	150000	110	54.55	29.00
	Ultra filtration	10	6000	60000	52	115.4	13.68
<i>Leuconostoc halophilus</i> FALB1	Culture supernatant	1000	8000	8000000	580	13.79	100
	Ammonium sulphate Precipitation	50	8000	400000	450	17.78	77.57
	Trichloroacetic acid Precipitation	25	8200	205000	266	30.83	45.90
	Ultra filtration	10	8200	82000	110	74.55	19.00

^ITotal activity was determined by the multiplication of volume by activity

^{II}Protein concentration was determined by the Bradford method

^{III}Specific activity is the activity units divided by the protein concentration (µg/ml)

^{IV}Recovery percentage is the remaining protein concentration as a percentage of the initial protein concentration

FCF: FCF : fermented corn flour, FALB: fermented African locust bean

Table 4. Characterization of bacteriocins produced by *Pediococcus halophilus* FCF7 and *Leuconostoc gelidum* FALB1

Isolates	Stability	Stability of bacteriocins at:		pH	Properties of bacteriocins	Bacteriocins
		Exposed Temperature (°C)	Storage Temperature (°C)			
<i>Pediococcus halophilus</i> FCF7	Stable	40,60	4	2,4,6	Broad spectrum, bactericidal, produced late in the growth cycle	pediocin
	Slightly stable	80,100	10	8,10		
	Unstable	121	24±1	12		
<i>Leuconostoc gelidum</i> FALB1	Stable	40,60,80	4	2,4,6	Broad spectrum, bactericidal, produced early in the growth cycle	leucocin
	Slightly Stable	100	10	8,10		
	Unstable	121	24±1	12		

FCF: FCF : fermented corn flour, FALB: fermented African locust bean

Table 5: Effects of Bacteriocins produced by Lactic Acid Bacteria against Test Isolates

	<i>P. halophilus</i> FCF7	<i>L. gelidum</i> FALB1
<i>E. coli</i> N2(mm)	6	8
<i>P. aeruginosa</i> N7 (mm)	6	8
<i>L. monocytogenes</i> W 6(mm)	-	8
<i>S.dysenteriae</i> N11(mm)	-	8
<i>B.cereus</i> W18 (mm)	6	8
<i>S. typhimurium</i> N8(mm)	6	8
<i>E. coli</i> W4 (mm)	5	8
<i>P. vulgaris</i> W7 (mm)	6	8
<i>K.aerogenes</i> N12(mm)	6	8
<i>S. aureus</i> N16b(mm)	6	8
<i>B. subtilis</i> N20 (mm)	6	8
<i>K.ozaenae</i> W24(mm)	-	8
<i>P. mirabilis</i> N16a(mm)	5	9

FCF : fermented corn flour, FALB: fermented African locust bean, N:nono, W:wara, mm:Millimeter

DISCUSSION

The fermented corn flour (FCF) and fermented African locust bean (FALB) analyzed contained lactic acid bacteria (LAB) in varying numbers. The presence of LAB in locally fermented foods has been reported by Oyeleke *et al.* (2006); Mohammed and Ijah (2013). The LAB pose no threat to the health of the individual that consume these products as reported by Mohammed and Ijah (2013). *Pediococcus halophilus* FCF 7 and *Leuconostoc gelidum* FALB 1 produces bacteriocin activity between 5800 AU/mL and 8000 AU/mL against the tested microorganisms, pH between 0.80 and 0.90. This is similar to the findings of Ogunbanwo *et al.*(2003). And in conformity with Mohammed *et al.* (2012). Lactobacilli are important in food fermentation as well as the production of bacteriocins. Bacteriocins are

responsible for inhibition of pathogenic and spoilage microorganisms in foods as reported by Adam and Moss (2008). The optimal bacteriocin recovery achieved was (19.00%) from *Leuconostoc gelidum* FALB 1 while the least bacteriocin recovery (13.68%) was obtained with *Pediococcus halophilus* FCF after ammonium sulphate precipitation, trichloroacetic acid precipitation and ultra-filtration analysis processes of purification. This is in conformity with the report of Invanova *et al.* (2000). Ultrafiltration experiments showed that bacteriocins were unable to pass through 1,000 and 10,000 KDA molecular weight cut off membrane. A tendency to aggregate with other proteins has been reported in bacteriocins produced by other lactic acid bacteria and might have contributed to why the bacteriocins could not pass through the membrane with low molecular weight cut-off. The bacteriocins produced by the bacteriocin producing LAB were heat stable at 20°C, 40°C and 60°C in most cases but slightly stable at 80°C, 100°C and unstable at 121°C for 30 minutes which is in consistent with Ogunbanwo *et al.*(2003). Bacteriocins produced by the LAB were also stable at 4°C, slightly stable at 10°C and unstable at 24±1°C storage temperatures in most cases. Temperature stability is important if the LAB is to be used as food preservative, because many procedures of food preparation involve heating steps. The activity of bacteriocins elaborated by the LAB was also observed to be pH dependent. The highest antibacterial activity was exhibited in an acidic pH range of 2 - 6, while inactivation occurred mostly at pH 8 - 12. Bacteriocins isolated from *Pediococcus halophilus* FCF 7 and *Leuconostoc gelidum* FALB 1 were shown to have high activity and stability at pH 2, 4 and 6 Slightly stable at pH 8,10 and unstable at pH 12 respectively against the range of pathogenic and spoilage microorganisms. This is similar to the report of Kacem *et al.* (2005). Bacteriocins produced by the bacteriocin producing LAB were identified as pediocin produced by *Pediococcus halophilus* FCF7 and leucocin produced by *Leuconostoc gelidum* FALB 1. Several types of bacteriocins from food-associated lactic acid bacteria have been identified and characterized, of which the important ones are nisin, diplococcin, acidophilin, bulgarican, helveticins, lactacins and plantaricins (Nettles and Bare-foot,1993). Large number of bacteriocins have been isolated and characterized from LAB and some have

acquired a status as potential antimicrobial agents because of their potential as food preservatives and antagonistic effects against important pathogens. Similarly, Soomro *et al.* (2002) and Kacem *et al.* (2005) had identified and characterized bacteriocins produced by *Lactococcus lactis* sub sp. *lactis* ATCC11454, *Pediococcus pentosaceus* FBB61, *Pediococcus acidilactici* H, *Leuconostoc gelidum* UAL187, *Lactobacillus helveticus* 481 and *Camobacterium piscicola* LV17 as Nisin, pediocin A, Pediocin AcH, Lecucocin, Helveticin J and Camobacteriocin respectively. This findings also agrees with Mohammed (2012) .This is also in conformity with Mohammed *et al.* (2012). The bacteriocins produced by *L.gelidum* FALB1 inhibited all test isolates while bacteriocins produced by *P.halophilus* FCF7 inhibited all the test isolates except *Listeria monocytogenes* W6, *Shigella dysenteriae* N11 and *Klebsiella ozaenae* W24. This agrees with earlier reports by Sanni *et al.* (1999) and Daeschel (2011).

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