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Antimicrobial Activities of Lactic Acid Bacteria Strains Isolated from Burkina Faso Fermented Milk

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Abstract: Eight strains of lactic acid bacteria producing bacteriocin were isolated from Burkina Faso fermented milk samples. These strains were identified to species: *Lactobacillus fermentum*, *Pediococcus spp.*, *Leuconostoc mesenteroides subsp. meseteroides, Lactococcus.* Isolated bacteriocin exhibited antibacterial activity against *Enterococcus faecalis* 103907 CIP, *Bacillus cereus* 13569 LMG, *Staphylococcus aureus* ATCC 25293, *Escherichia coli* 105182 CIP using the agar drop diffusion test. The inhibition diameters obtained with bacteriocin are between 8 mm and 12 mm. Gram-positive indicator bacteria were most inhibited. The activities of the bacteriocin were lost after treatment with all the proteolytic enzymes (α-chymotrypsin, trypsin, pesin), whereas treatment with lipase, catalase, α-amylase did not affect the activity of the bacteriocin.

Key words: Bacteriocin, lactic acid bacteria, fermented milk

Introduction

Lactic acid bacteria produce various compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocin or bactericidal proteins during lactic fermentations (Talarico and Dobrogosz, 1989; Lindgren and Dobrogosz, 1990; Piard and Desmazeaud, 1991; Anderssen *et al*., 1998; Sholeva *et al*., 1998; Ouwehand, 1998; Zhennai, 2000; Oyetayo *et al*., 2003).

The bacteriocins from the generally recognized as safe (GRAS) lactic acid bacteria (LAB) have arisen a great deal of attention as a novel approach to control pathogens in food-stuffs.

Bacteriocins are antimicrobial proteinaceous compounds that are inhibitory towards sensitive strains and are produced by both Gram-positive and Gram-negative bacteria (Tagg *et al*., 1976).

The antimicrobial effect of lactic acid bacteria has been appreciated by man for more than 10000 years and has enabled him to extend the shelf life of many foods through fermentation processes.

Innovative approaches have been tried as alternative to antibiotics in treating gastrointestinal diseases and these include using live biotherapeutic agent such as bacterial isolates (Daly and Davis, 1998; Soomro *et al*., 2002; Oyetayo *et al*., 2003)

Lactic acid bacteria exert strong antagonistic activity against many microorganisms, including food spoilage organisms and pathogens. In addition, some strains may contribute to the preservation of fermented foods by producing bacteriocins (Brink *et al*., 1994).

Research on Bacteriocins from lactic acid bacteria has expanded during the last decades, to include the use of bacteriocins or the producer organisms as natural food preservatives.

The lactic acid fermentation, which these bacteria perform has long been known and applied by the humans for making different food stuffs.

The rural people in Burkina Faso still produce unpasteurized fermented milk by traditional methods, since such milk products still enjoy loyal following in rural communities.

Fermented milk play important role in the diet of low income and the majority of people living in the rural areas of Burkina Faso; fermented milk still produced using primitive utensils.

To our knowledge information does not exist on the traditional fermentative micro flora producing antibacterial substances that Fulani tribe used to produce fermented milk in Burkina Faso.

The aim of this study was the screening of antimicrobial activities among lactic acid bacteria from fermented milk.

Materials and Methods

Bacteria strains and media: The eight strains of lactic acid bacteria were isolated from Burkina Faso fermented milk samples (Savadogo *et al*., 2004a). Fermented milk samples from thirty cow's and goat's were collected from individual households of rural areas in northern Burkina from July 1999 to January 2000.

The strains were stored at - 80°C in MRS (De Man *et al*., 1960) broth medium containing 250 ml glycerol / L.

Before experimental use the cultures were propagated twice in MRS at 37°C the transfer inoculum was 1% (v/v) of 16 h culture grown in fresh medium.

Table 1: Inhibition of various indicator organisms by bacteriocin produced by our lactic acid bacteria

Lactic acid bacteria strains	Indicator organisms inhibited	Diameter of inhibition (mm)
S1	*Enterococcus faecalis* 103907 CIP	12
S2	*Bacillus cereus* 13569 LMG	10
S3	*Enterococcus faecalis* 103907 CIP	9
	Staphylococcus aureus ATCC 25293	9
	Escherichia coli 105182 CIP	9
S4	*Enterococcus faecalis* 103907 CIP	10
S5	*Enterococcus faecalis* 103907 CIP	8
	Staphylococcus aureus ATCC25293	10
	Escherichia coli 105182 CIP	8
S6	*Staphylococcus aureus* ATCC 25293	9
S7	*Enterococcus faecalis* 103907 CIP	9
S8	*Staphylococcus aureus* ATCC 25293	10

Table 2: Morphological, cultural and physiological characteristics of isolated strains (Savadogo *et al.*, 2004a)

Strains	Cell form	Cellular arrangement	Growth in 6.5% NaCl	Gram	Catalase	Gas from glucose and sporulation
S1	Cocobacilli	single and chains	-	+	-	-
S2	Ovoid	single	-	+	-	-
S3	Cocobacilli	Single and chains	-	+	-	-
S4	Spherical	Pairs and tetrad	-	+	-	-
S5	Spherical	Pairs and chains	-	+	-	-
S6	Ovoid	Pairs and tetrad	-	+	-	-
S7	Spherical	single and chains	-	+	-	-
S8	Rods	single and chains	-	+	-	-

+ = positive, - = negative

Indicator bacteria strains: The microorganisms used were: *Enterococcus faecalis* 103907 CIP, *Bacillus cereus* 13569 LMG, *Staphylococcus aureus* ATCC 25293, *Escherichia coli* 105182 CIP.

Extraction of bacteriocins: The lactic acid bacteria strains were propagated each in 1000 ml MRS broth (pH 7,0). For extraction of bacteriocin, a cell-free solution was obtained by centrifuging (10,000 rpm for 20 min, at 4°C) the culture and was adjusted to pH 7.0 by means of 1M NaOH to exclude antimicrobial effect of organic acid. The cell-free solution obtained was precipiced with ammonium sulphate (40% saturation). The mixture was stirred for 2 h at 4°C and later centrifuged at 20,000 rpm for 1 h for 4°C. The precipitates were resuspended in 25 ml of 0.05 M potassium phosphate buffer (pH7.0). The new precipitate were collected and used in a disk diffusion assay.

Determination of bacteriocin activity: A disk diffusion assay procedure was used (Tagg and McGiven, 1971). Aliquot of 50µl from each (extract) were placed on the disk. The plates were incubated overnight at 37°C (aerobically) for the indicators pathogens bacteria (Table 1).

All the tests were performed in Mueller-Hinton agar (Becton Dickinson, USA). Mueller-Hinton agar was inoculated (inundation method) with pathogen indicator bacteria ($5X10^5$ CFU/ml). Overnight cultures of each indicator strain were used. The agar inoculated was incubated aerobically at 30°C (Gram-negative) or 37 °C (Gram-positive) for 24 h.

The diameters of the inhibition zone were measured (Rammelsberg and Radler, 1990).

Enzyme treatments: Bacteriocin was asked for its sensitivity to various enzymes.

Enzymes (all obtained from sigma) and their respective buffers were lipase (8.6 U/mg) in 0.05 Tris hydrochloride (pH 8.0) 0.01 M $CaCl_2$; α-chymotrypsin (47 U/mg) in 0.05 M tris hydrochloride (pH 8.0) 0.01M $CaCl_2$; trypsin (Type x, 15000 U/mg), in 0.05 M Tris Hydrochloride (pH 8.0); Pepsin (3.2 U/ml) in 0.2M citrate (pH 6.0); Catalase (2.0 U/mg) in 10 mM potassium phosphate (pH 7.0); α-amylase (1000 U/mg) in 1N NaOH (pH 6.5). Samples of bacteriocins (500 µl) were incubated with 500 µg of each enzymes per ml for 60 min at 37°C except for samples containing trypsin, α-chymotrypsin and catalase which were incubated at 25°C.

Results and Discussion

Isolated strains characteristics: The characteristics of all the strains are noted in Table 2 (Savadogo *et al.*, 2004a). These characteristics are common to lactic acid bacteria ones (Axellsson, 1993; Krieg, 1984 ; Roissart and Luquet, 1994).

Table 3 : Diamètres d'inhibition après hydrolyze enzymatic

LAB strains	Indicators strains	Trypsin	Chymotrypsin	Pepsin	Lipase	Amylase	Catalase
S1	*Enterococcus faecalis* 103907 CIP	0	0	0	12	11	11
S2	*Bacillus cereus* 13569 LMG	0	0	0	10	10	10
S3	*Enterococcus faecalis* 103907 CIP	0	0	0	9	9	9
	Staphylococcus aureus ATCC 25293	0	0	0	9	9	9
	Escherichia coli 105182 CIP	0	0	0	8	9	8
S4	*Enterococcus faecalis* 103907 CIP	0	0	0	10	9	9
S5	*Enterococcus faecalis* 103907 CIP	0	0	0	8	7	8
	Staphylococcus aureus ATCC 25293	0	0	0	9	9	9
	Escherichia coli 105182 CIP	0	0	0	8	8	7
S6	*Staphylococcus aureus* ATCC 25293		0	0	9	9	9
S7	*Enterococcus faecalis* 103907 CIP		0	0	9	9	9
S8	*Staphylococcus aureus* ATCC 25293		0	0	10	9	10

LAB: Lactic Acid Bacteria

Strains S1 and S3 were identified as *Lactobacillus fermentum*, Strains S2, S4, S6 were identified as *Pediococcus spp.*, the strain S5 was identified as *Leuconostoc mesenteroides subsp. mesenteroides*. Strains S7, S8 were identified as *Lactococcus sp.* (Savadogo *et al.*, 2004a).

These results show a certain heterogeneousness of fermented milk of Burkina. Savadogo *et al.* (2004ab) showed that fermented milk of Burkina Faso were rich in various genus of EPS producing lactic bacteria among which we can quote *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Lactococcus*.

The heterogeneousness of the lactic bacteria in fermented milk was also observed by the other authors who one worked on fermented milk, notably Yodoamijoyo *et al.* (1983) in Idonesie, Hamama (1992) in Morocco, Isono and his co-workers (1994) in Tanzania, Beukes *et al.* (2001) in South Africa. In a general way the genus usually met in traditional fermented milk are *Lactobacillus, Leuconostoc, Lactococcus, Streptococcus, Pediococcus*.

The heterogeneousness of the lactic bacteria observed would contribute to the hygienic quality and organoleptic of the milk fermented traditionally, that justifies the importance of his consumption .

Production of bacteriocin by isolated strains: The extracts of eight strains of lactic acid bacteria gave zones of inhibition onto the indicator pathogenic strains tested. The Table 1 gives the results of inhibition (inhibition diameter), indicators strains inhibited are *Escherichia coli* 105182 CIP *Enterococcus faecalis* 103907CIP, *Staphylococcus aureus* ATCC 25293, *Bacillus cereus* 13569 LMG. The diameters of inhibition are included between 8 mm and 12 mm. The biggest diameter of 12 mm inhibition [photo 1] is obtained with the extract of strain S1 (*Lactobacillus fermentum*) on *Enterococcus faecalis,* as for the smallest diameter is obtained with the extract of strain S5 (*Leuconostoc mesenteroides*) on the same indicator strain *Enterococcus faecalis*. The most inhibited indicators strains are the most part of gram positive bacteria (*Enterococcus faecalis* 103907CIP, *Staphylococcus aureus* ATCC 25293, *Bacillus cereus 13569 LMG* [photo 2]), a single gram negative indicator bacteria (*Escherichia coli* 105182 CIP) was inhibited by the extracts of bacteriocins. Gram positive indicator bacteria is much more sensitive to bacteriocin of our lactic acid bacteria strains than gram negatif indicator bacteria.

These results indicate us that some of our lactic acid bacteria strains are capable of synthesizing inhibitive substances of pathogenic bacteria, also these inhibitive substances produced by our lactic acid bacteria strains act differently on the pathogenic reference indicators strans. Inhibitive substances produced by the lactic acid bacteria can be generally protein (Klaenhammer, 1993; Jimenez-Diaz *et al.*, 1993; Vandenbergh, 1993).

However the importance of the inhibition effect varies according to serotypes. The gram positive pathogenic bacteria are the most sensitive to the bacteriocin produced by the lactic acid bacteria. The resistance of gram negative bacteria is attributed to the particular nature of their cellular envelope, the mechanisms of action described for bacteriocin bringing in a phenomenon of adsorption. According to Bhunia *et al.* (1991) the pediocin (bacteriocin produced by *Pediococcus acidilactici*) interacts with lipoteichoic acids absent in gram negative bacteria. These molecules would play the role of site of necessary not specific reception to produce the bactericidal effect. Earlier reports (Tagg *et al.*, 1976; Daeschel and Klaenhamner, 1985; Muriana and Klaenhamer, 1991; Sanni *et al.*, 1999) have shown that some bacteriocins produced by gram-positive bacteria have a broad spectrum of activity. These variations of sensibility are due to the characteristics of indicators strains (presence or absence of receiving sites or immunoprotein) and thus in level of hurts caused by the inhibitive factor.

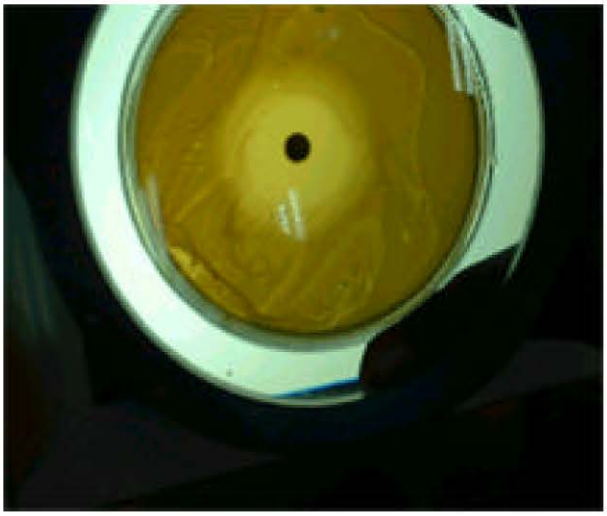

Photo 1: Inhibition of *Enterococcus faecalis* 103907 CIP by strain S1 bacteriocin

Photo 2: Inhibition of *Bacillus cereus* 13569 LMG by strain S2 bacteriocin

The known bacteriocin (nisin for example) does not still act on the sorts taxonomic close.

Nisin has an inhibitory effect against a wide variety of gram-positive food-borne pathogens and spoilage microorganisms (Rodriguez, 1996) and can also act on several gram-negative bacteria when the integrity of their outer membranes is disrupted (Kordel and Sahl, 1986; Stevens *et al.*, 1991). The use of nisin as a food preservative dates back to 1956, when nisin was proposed to control growth and spore formation of *Clostridium botulinum* and *Clostridium sporogenes* in cheese (Mattick and Hirsch, 1956). Nisin is the only bacteriocin that has been approved by the World Health Organization as a preservative in food (Vandenbergh, 1993), and Nisalpin, the commercial product containing 2.5% pure nisin A, is being legally used in more than 50 countries for specific food applications (Delves-Broughton and Friis, 1998). However, the loss of nisin activity from the commercial form has been reported for several food products during storage (Delves-Broughton, 1990). Moreover, the use of nisin in its free form in cheese can be expensive and results in inhibitory effects against the suitable acidifying or aroma-producing starter cultures, decreasing growth and acidification (Roberts, 1991). An alternative to the addition of free nisin to fermented food systems is the use of nisin-producing strains during fermentation processes (Maisner-Patin *et al.*, 1992; Roberts *et al.*, 1992). However, bacteriocin-producing organisms in cheese making can cause alterations in the cheese-making process, such as delayed acidification of the curd with a concomitant increase in residual lactose (Fox *et al.*, 1996; Roberts, 1991).

Diameters of inhibition after enzymatic hydrolysis: The results of the Table 3 show that antibacterial compounds produced are inactive by all the proteolytic enzymes (pepsin, trypsin, chymotrypsin), indicating that the inhibitory compounds are proteinaceous nature, a general characteristic of bacteriocin. No zone of inhibition was discovered after stake in the presence of our extracts with these various enzymes, knowing that our initial extracts produced zones of inhibition on some tested pathogenic indicators strains.

Inhibitive substances of our lactic acid bacteria strains could be proteinaceous antibacterial compounds. So the haste in the sulfate of ammonium during the extraction was steered against protein substances, the enzymatic action comes to confirm so the specificity of the haste in the sulfate of ammonium.

On the other hand no inactivation is noticed in the presence of amylase, catalase and lipase. This report can explain by the fact that molecules responsible for the inhibition cannot glucidique or lipidique or still the inhibition results of peroxide of hydrogen. Our extracts (substances) are thus of protein nature, some molecules of sugars, or lipids could join to these proteins; whatever these molecules glucidiques and lipidiques are not indispensable for the inhibition.

Following the works on colicines (bacteriocin from Gram negative bacteria), Tagg *et al.* (1976) quote five required criteria so that a chemical substance is called bacteriocin: the presence of a biologically active part of protein nature, a spectrum of inhibitive activity narrow and centred on the homologues, a mode of bactericidal action, the adsorption to specific receivers and nature plasmodic genetic determiners coding for the production of the bacteriocin and for the immunity in this one.

The studies realized by Klaenhammer (1993) on *Lb.brevis* DSM9296 on one hand looked 9 mm of

diameter of inhibition with *E. faecalis*, 16 mm of diameter of inhibition with *Streptococcus xylosus*; on the other hand on *Lc. lactis 99* looked 15 mm of diameter of inhibition with *Bacillus linens* SR3, 10 mm of diameter of inhibition with *Streptococcus xylosus*, 4 mm onto *Staphylococcus aureus*.

Jimenez-Diaz *et al.* (1993) showed that the activity antibacterial of the floating of *Lb. Plantarum LP* co10 was not only eliminated by the action of proteases but also by the action of enzymes glycolytic and lipolytic.

Conclusion: The cell-free supernattants from eight strains of lactic acid bacteria exhibited antimicrobial activity. The potential application of bacteriocins as consumer friendly biopreservatives either the form of protective cultures are as additives is significant besides being less potentially toxic or carcinogenic than current antimicrobial agents, lactic acid bacteria and their byproducts have been shown to be more effective and flexible in several applications. Many bacteriocins of LAB are safe and effective natural inhibitors of pathogenic and food spoilage bacteria in various foods. Antimicrobial compounds produced by LAB have provided these organisms with a competitive advantage over other microorganisms.

These researches are all vital in the sense that functional properties in lactic acid bacteria improve preservative effect and add flavor and taste.

Lactic acid bacteria have an essential role in most food and beverage fermentation processes, one of the earliest known food preservation of fermented foods and beverage.

These isolated strains can positively have impact on their use as starter cultures for traditional fermented foods, with a view to improving the hygiene and safety of fermented milk so produced.

The further studies will be focused on the characterization of amino acid and nucleotide sequences of these antibacterial compounds.

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