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Bacteriocins from lactic acid bacteria inhibit food borne pathogens.

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Abstract*: Lactic acid bacteria were isolated from naturally fermenting food products (ogi, nunu and soybean). They were screened for their ability to produce bacteriocins and inhibit two pathogenic organisms. Twenty four lactic acid bacteria were isolated. Eight were catalase negative. Their probable identities were: Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus acidophilus, Leuconostoc lactis, Pediococcus pensaceus, Leuconostoc mesenteriodes and organism F (identity not confirmed). Extracellular extracts of the eight isolates inhibited pathogenic strains of Escherichia coli (ATCC 117755) and Staphylococcus aureus (ATCC 12600). Inhibition zones ranged from 9.67 to 19.33 mm against E. coli and 7.33 to 15.00 mm against S. aureus. L. acidophilus and the control (subtilisin) had the highest inhibition zones (15 mm) against S. aureus, while L. fermentum had the highest inhibition zone (19.33 mm) against E. coli. After treatment with catalase, the inhibition zones for L mesenteroides, P pentosaceus and L. lactis against S.aureus became virtually non-existent (0.3-1.0 mm) indicating that the inhibitory substance was of a non-protein nature. The same trend was observed for the inhibitory effects of these organisms on E.coli (inhibitory zones of 0.00 to 0.67 after treatment with catalase). L acidophilus, L. plantarum and L. fermentum produced bacteriocin. Their inhibition zones were maintained after treatment with catalase but became non-existent after treatment with proteinase K.*

Keywords: *food borne pathogens, inflammatory diseases, bacteriocins, lactic acid bacteria*

I. Introduction

Lactic acid bacteria are industrially important organisms recognized for their fermentative ability, health and nutritional benefits. Species used for food fermentations belong to the genera *Lactococcus, Streptococcus, Pediococcus, Leuconostoc, Lactobacillus,* and the newly recognized *Carnobacterium.* Lactic acid bacteria (LAB) occur naturally in several raw materials like milk, meat and flour. They are used to produce many fermented foods [1]. One important attribute of lactic acid bacteria is their ability to produce antimicrobial compounds such as organic acids, diacetyl, hydrogen peroxide, ethanol, reuterin and bacteriocins or bactericidal proteins [2, 3]. In recent years, interest in bacteriocins has grown substantially due to their potential usefulness as natural food preservatives in addition to promoting good health [3]. Selected strains of lactic acid bacteria, used as starter culture, may inhibit spoilage microorganisms and pathogens by production of metabolites with anti microbial properties [4]. Different lactobacilli, bifidobacteria, or probiotic mixtures have been shown to alleviate digestive diseases in experimental animals [5, 6) and in inflammatory bowel diseases in humans [7, 8, 9] Pathogenic *Escherichia coli* induce acute inflammatory responses while *Staphylococcus aureus* elicit mild inflammatory responses that may be more persistent [10]. Bacteriocins produced by lactic acid bacteria are defined as extracellular primary or modified products of bacterial ribosomal synthesis, which can have relatively narrow spectrum of bactericidal activity [11]. Health benefits currently being investigated in favor of probiotic microorganisms include their role in alleviating chronic intestinal inflammatory diseases; prevention and treatment of pathogen induced diarrhea; urogenital infections and atopic diseases[12,13,14] Bacteriocin producing strains can be used as part of an adjunct to starter cultures for fermented foods in order to improve safety and manage health more effectively [15]. Several bacteriocins with industrial potential have been purified and characterized. Bacteriocins of lactic acid bacteria, according to the classification are divided into four classes [16].. Nowadays, the consumer pays better attention to the relation between food and health [17]. The use of functional starter cultures in food fermentation tailor made for increased bacteriocin production is being explored for improved healthcare and disease management [18]. The target of bacteriocins is usually the cytoplasmic membrane. There is need to improve bacteriocin production in Lactic acid bacteria for health benefits. The present research is a preliminary attempt to ascertain bacteriocin production by different strains of lactic acid bacteria and their efficacy against the antagonistic affects of pathogenic bacteria. In course of the research, lactic acid bacteria will be isolated from selected fermented foods (ogi, nunu and soybean) and tested for their bacteriocin producing potentials. Those with good potentials could serve as adjuncts for accelerated food fermentations to reduce and manage inflammatory diseases caused by food borne pathogens.

II. Materials and methods

2.1 Preparation of samples used for the isolation of lactic acid bacteria

Ogi: The ogi used for this research was prepared traditionally according to the method of [19]. The grains of *Zea mays* were cleaned and steeped in water for 2 days in a pot. The water was decanted and grains wet-milled before sieving with muslin cloth. The pomace was discarded and the starch suspension was allowed to sediment and fermentation was carried out for 3 days during which the fermenting natural flora was isolated.

Soybean: Thirty (30) grams of soybean seeds were soaked in 150ml of water. The water was changed at intervals to reduce microbial growth on the seeds. After steeping for eight hours, 10 grams was pounded and added into 90 ml of 0.1% sterile peptone water, incubated for 24 hours at 30^{o}C and used for isolation of microorganisms.

Nunu**:** Ten (10) grams of fresh nunu (naturally fermenting cow milk) was added into 90ml of 0.1% sterile peptone water, incubated for 24 hours at 30^{o}C and the fermenting microorganisms were isolated**.**

2.2. Isolation and screening for bacteriocin-producing lactic acid bacteria from Ogi, soybean and nunu:

Isolation and screening of bacteria-producing lactic acid bacteria was done as described by Todorov *et al.* [19] with little modification. One milliliter of each of the prepared samples from ogi, soybean and nunu was innnoculated into 9 ml sterile De Man Ragosa and Sharpe (MRS) broth (Fluka, UK) for enrichment of the resident LAB. The tubes were incubated at 30^{o}C for 48 hours. The aliquots (1ml) of the culture from each of the tubes were diluted serially to 10^{10} times and 1 ml was spread evenly on MRS agar plates. The plates were incubated at 30^{o}C for 16 hours till colonies appeared.

The indicator or target organisms (*Escherichia coli*, ATCC 117755 and *Staphylococcus aureus*, ATCC 12600) were grown overnight in nutrient broth. A volume of 1 ml of the indicator organisms, with a total of approximately 5 x 10^5 cfu/ml were seeded into 15 ml of semi-solid MRS agar. After agitation they were poured over the plates containing MRS agar on which the strains under test had grown. The plates were incubated at 30^{o}C for 24 hours and checked for inhibition zone. Inhibition was considered positive when the inhibition halo of the indicator strains above the LAB colonies were more than 2 mm and such bacteria were considered as potential bacteriocin producers. Such colonies were carefully collected and transferred into MRS broth (different tubes for each colony). The colonies were purified by plating on MRS agar. This procedure was repeated in order to further purify the isolates. The purified colonies were maintained on MRS agar slants for immediate use and in 20% glycerol for storage at 20^{o}C [20].

2.3 Identification of the isolates:

Isolates obtained from the screening test were identified by conventional microbiological and biochemical procedures, which included microbial examination of cell morphology and biochemical tests (gram reaction, catalase test, mobility test, arginine test, growth at 4%, 6.5% NaCl for 72 hours at 30^{o}C and sugar fermentation tests). The sugars used were glucose, L-arabinose, dextrose, D-fructose, D-xylose, galactose, raffinose, sorbitol, and sucrose**.**

2.4 Determination of the inhibition zone of the isolates at 30^{o}C for 72 hours against *Staphylococcus aureus* ATCC 12600, and *E. coli* ATCC 117755 using agar well diffusion test

Eight isolates were tested for antimicrobial activity. One milliliter of the indicator strains (with approximately 5x10^5 cfu/ml) cultured in nutrient broth at 30^{o}C for 18 hours was seeded into 15ml of semi-solid nutrient agar, and Mackonkey agar (for ATCC 12600 and ATCC 117755 respectively). After solidification of the agar, a well of 5 mm diameter was aseptically cut into it and 1 ml of the fluid from the cultured strains under test (for antimicrobial activity) obtained as indicated above was added to each well. The plates were kept at 47^{o}C for 4 hours to ensure diffusion of the fluid into the agar and examined for inhibition after incubation at 30^{o}C for 24, 48 and 72 hours [21].

2.5 Preparation of the cell-free supernatant:

The strains that possessed antimicrobial activity but had negative result for hydrogen peroxide were used to prepare the cell-free supernatant. The strains were incubated in MRS broth (Fluka) at 30^{o}C for 24 hours. Subsequently, the pH of the culture was adjusted to 6.5 with sterile 1M NaOH. The culture was then centrifuged at 3000g for 15 minutes and sterilized by filtration through a 0.22µm pore size membrane filter (Whatman).

2.6 Sensitivity of the cell-free supernatant (bacteriocin-like substance) to enzyme (catalase and protenase K):

One milliliter of sterile cell-free supernatant obtained as described above were incubated for 2 hours at 37^{o}C in the presence of 1 mg/ml catalase (Fluka) and proteinase K (Sigma) and catalase and later inactivated in a water bath for 10 minutes. Control experiments used were cell-free supernatant without enzyme treatment and 1 mg/ml of subtilisin. Antimicrobial activity of each of the isolates was determined by agar well diffusion test with *E. coli* ATCC 117755 and *Staphylococcus aureus* ATCC 12600 as indicator organisms.

III. Results

3.1 Identification of the isolates

Ten (10), six (6) and eight (8) strains of lactic acid bacteria were isolated from ogi, nunu and soybean respectively. After screening to eliminate catalase positive organisms, only eight isolates remained. Table 1 shows the characteristics of these eight isolates. The colony morphology and biochemical characterization of the isolates were studied. Majority of the strains were rods while some were cocci. *Lactobacillus fermentum, Lactobacillus acidophilus* (two of the isolates), *Pediococcus pentasecens* were able to liberate ammonia from arginine, while *Lactobacillus plantarium, Leuconostoc mesenteriodes* and *Leuconostoc lactis* were not able to liberate ammonia from arginine.

All the isolates were able to grow at 15^{o}C, 4% and 6.5% NaCl and were able to utilize glucose to produce acid but only three (*Lactobacillus fermentum, Lactobacillus acidophilus* and *Lactobacillus plantarum*) were able to produce gas in addition to the acid. *Lactobacillus fermentum* was able to utilize all the fourteen sugars. Dextrose, D-fructose, galactose, mannose, melebiose were utilized by all the isolates. Organism F displayed a peculiar characteristic by producing gas bubbles in seven out of the fourteen sugars used for the test .The probable identity of this isolate could not be determined using normal biochemical tests.

3.2 Effect of period of incubation on the inhibition zone of the isolates against *Staphylococcus aureus*

Table 2 shows the inhibition zones of test organisms against *Staphylococcus aureus* at varying incubation periods. There were no significant differences ($p>0.05$) in the inhibition zones of *Lactobacillus fermentum, Lactobacillus acidophilus, Leuconostoc mesenteriodes* and *Leuconostoc lactis* between 24 and 72 hours of incubation. For *Lactobacillus plantarum*, there was no observable inhibition zone after 72 hours. The degree of inhibition of *Lactobacillus plantarum* against *S. aureus* was not significantly different ($p>0.05$) within the first 48 hours.

3.3 Effect of catalase and proteinase K enzymes on the antimicrobial properties of some lactic acid bacteria against *Escherichia coli* (ATCC 117755) and *Staphylococcus aureus* (ATCC 12600)

The antimicrobial activities exhibited by *Lactobacillus fermentum, Lactobacillus acidophilus* and *Lactobacillus plantarum* against *Staphylococcus aureus* (ATCC 12600) were found to be insensitive to the action of catalase enzyme. There was no significant reduction in the inhibition zone exhibited by these isolates (Table 5). On the other hand, the antimicrobial activity exhibited by these organisms against *Staphylococcus aureus* was found to be sensitive to the action of protease enzyme. Thus, incubation of the extracellular extract obtained from these isolates together with proteinase K elicited a significant loss ($p<0.05$) of the antimicrobial activity, as determined by the agar well diffusion test.

There was significant reduction in the antimicrobial activity of *Leuconostoc lactis, Pediococcus pentasaceus, Leuconostoc mesenteroides* and organism F "not determined" (Table 5) after treatment with catalase enzyme. No significant reduction in the inhibition zone was observed after treating the extracellular extract of these isolates with proteinase K using *Staphylococcus aureus* as the target organism.

Subtilisin (a known bacteriocin) was used as the control and no significant reduction in the antimicrobial activity was observed after treatment with catalase. Meanwhile, significant inactivation in the antimicrobial activity of subtilisin was recorded after treatment with proteinase K.

3.4 Effect of different enzymes (catalase and proteinase K) on the inhibitory property of some lactic acid bacteria against *Escherichia coli* (ATCC 117755)

Treatment with catalase did not alter the antimicrobial activities of the extracellular extract of *Lactobacillus acidophilus, Lactobacillus plantarum* and *Lactobacillus acidophilus* against *E. coli*. Complete inactivation or significant reduction in the antimicrobial activity was observed after treatment of the extracellular extract of same organisms with proteinase K (Table 6).

Complete inactivation or significant reduction in antimicrobial activity was observed after treatment of the cell-free supernatant of *Leuconostoc mensenteriodes*, organism F, *Pediococcus pentosaceus* and *Leuconostoc lactis* with catalase. The antimicrobial activity of the extracellular extract of same organisms was not affected significantly ($P>0.05$) after treatment with proteinase K. No significant reduction in the antimicrobial activity of subtilisin (control) was observed after treatment with catalase while significant reduction was observed in the antimicrobial activity of subtilisin (control) after treatment with proteinase K.

IV. Discussion

The identification test carried out on the food isolates indicated the dominance of lactic acid bacteria in the ogi, nunu and soybean. Similar result was obtained by Odunfa and Adeyele [20]. Incubation period did not affect the antimicrobial activity of the isolates for at least 72 hours except for *Lactobacillus acidophilus*. The isolates had progressive growth until the 66th hour except for *Lactobacillus fermentum*. The sharp decrease in

pH of the isolates during 6-18 hours of incubation in MRS broth was due to the production of acids especially lactic acid. Production of acids has been reported to be higher during the stationary phase.

The antimicrobial activity of the lactic acid bacteria may be due to a number of factors. Among these are decreased pH levels, competition for substrate, and the production of substances with bacteriocidal or bacteriostatic action including bacteriocin [22]. In fact, the drop in pH arising from the production of lactic is enough to inhibit certain strains. This is because the non-dissociated form of lactic acid triggers a lowering of the internal pH of the cell that causes a collapse in the electrochemical proton gradient in sensitive bacteria, hence a bacteriostatic or bacteriocidal effect [23]. Probiotic bacteria have been observed by other researchers to have a beneficial effect on intestinal inflammation. Menard *et al*.(26] observed inhibition of polysaccharide induced secretion of Tumor Necrosis Factor (TNF-α) by *Streptococcus thermophilus* and *Bifidobacterium breve*. All the eight strains that showed negative result during catalase test inhibited the reference strains (*E. coli* ATCC 117755 and *S. aureus* ATCC 12600). The use of sterile base (1M NaOH) and catalase (Sigma) was to exclude any antimicrobial action arising from organic acids and from hydrogen peroxide respectively [21]. The antimicrobial activities of all the strains were not altered after excluding the possibility of organic acid as the cause of the inhibition.

When the neutralized extra-cellular extract from all the isolates was treated with catalase (Sigma), only four (neutralized extracellular extract from *Lactobacillus fermentum, L. acidophillus* and *L. plantarum, L. acidophillus*) out of the eight retained their microbial activity. This shows that hydrogen peroxide may have been the cause of antimicrobial activity exhibited by *Leuconostoc mesenteroides, Pediococcus pentasaceus, Leuconostoc lactis* and organism F (identity not confirmed).

The differences in inhibition zones and patterns observed for *E.coli* and *S. aureus* support observations that pathogenic organisms elicit responses in their host through different mechanisms. In humans, E. coli activates the TLR4 receptor while peptodiglycans found in gram positive bacteria such as *S. aureus* elicit inflammatory responses through activation of TLR2 receptor [25]. The ability of bacteria to establish infection is mediated in part by the ability of the host to respond to the invading organism [26]. The differences in responses of *E. coli* and *S. aureus* to different lactic acid bacteria maybe attributed to the nature and observable symptoms associated with their pathogenic effects [27,28]

Complete inactivation or significant reduction in antimicrobial activity of the neutralized extracellular extract of *L. acidophillus, L. plantarum, L. fermentum* and *L. acidophillus* was observed after treatment with proteinase K (Fluka, UK), confirming their proteolytic nature [28]. The cell free supernatant of the isolates lost their antimicrobial activity after treatment with proteinase K. This shows that out of the three Nigerian indigenous foods, nunu and ogi had better potentials for producing lactic acid bacteria with bio-preservative effects and health benefits (bacteriocin). The differences in the inhibition zones before and after treatment with catalase and proteinase K suggest that different responses were elicited by different bacteria pathogens.

V. Conclusion

Preventive health care can be effectively enhanced by the intake of foods that discourage pathogens that cause infections. Lactic fermentation with organisms like *Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus fermentum* promote wholesomeness of foods and health of individuals by discouraging the growth of pathogenic bacteria like *E. coli* and *S aureus*. Some of the inhibitory metabolites from the lactic acid bacteria are susceptible to proteolysis suggesting that they are proteins and might be bacteriocins.

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Table 1 Identity of the Isolates

Isolates	Morphology	Gram staining reaction	Catalase test	Arginine test	Growth at 15°C	Growth at 4% NaCl	Growth at 65% NaCl	Acid and gas from glucose	L+ Arabinose	Dextrose	D-fructose	D-xylose	Galactose	Lactose	Maltose	Mannitol	Mannose	Melebiose	Raffinose	Sorbitol	Sucrose	Probable organisms
A.	Rod	+	-	+	+	+	+	+ gas	+	+	+	+	+	+	+	+	+ gas	+	+	+	+	*L. fermentum*
B.	Rod	+	-	+	+	+	+	+ gas	-	+	+	+	+	+	-	-	+ gas	+	+	+	+	*L. acidophilus*
C.	Rod	+	-	-	+	+	+	+ gas	+	+	+	+	+	+	w	+	+	+	+	+	+	*L. plantarum*
D.	Rod	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+ gas	+	+	+	+	*L. plantarum*
E.	Rod	+	-	-	+	+	+	+	+	+	+	+	+	+ gas	+	+	+	+	+	+	+	*Leuconostoc mesenteroides*
F.	Rod	+	-	+	+	+	+	+	+	+	+	+	+ gas	+ gas	-	+	+ gas	+ gas	+ gas	+ gas	+ gas	*N. D.*
G.	Cocci	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+ gas	+	w	-	+	*Pediococcus pentosaceus*
H.	Rod	+	-	-	+	+	+	+	+	+	+	-	+	-	+	-	+	+	-	-	-	*Leuconostoc lactis*

+ = acid production; - = no acid production; N.D. = Not determined; w = weak acid production.

Table 2 Inhibition zone of some lactic acid bacterial against *Staphylococcus aureus*

Organisms	**24 (h) inhibition zone (mm)**	**48 (h) inhibition zone (mm)**	**72 (h) inhibition zone (mm)**	**SEM**
Lactobacillus fermentum	13.33[a]	16.00[a]	13.67[a]	0.69
Lactobacillus acidophilus	14.67[a]	16.33[a]	13.67[a]	0.89
Lactobacillus plantarum	15.67[a]	18.33[a]	ND	3.01
Lactobacillus acidophilus	18.00[a]	20.67[a]	15.67[a]	1.01
Leuconostoc mesenteroides	12.67[a]	16.33[a]	14.67[a]	1.04
OrganismF(Not fully characterized)	5.67[b]	8.00[ab]	10.00[a]	0.77
Pediococcus pentosaceus	12.67[ab]	21.00[a]	21.67[a]	1.97
Leuconostoc lactis	18.67[a]	23.00[a]	20.67[a]	1.05

a,b = means with different superscripts in the same row are significantly different at ($P<0.05$)
SEM = standard error of means
ND = not determined

Table 4.Inhibition of *Escherichia coli* by Lactic acid Bacteria

Organisms	Inhibition Zone(mm)			
	24h	48h	72h	SEM
Lactobacillus fermentum	11.67	13.66	ND	2.21
Lactobacillus acidophilus	18.00	20.33	18.00	0.75
Lactobacillus plantarum	14.00	17.67	20.33	1.63
Leuconostoc mesenteroides	18.00	20.00	18.00	1.33
Pediococcus pentosaceus	12.00	20.00	18.00	1.30
Leuconostoc lactis	19.00	22.00	22.33	0.65

Table 5 Effect of different enzymes (catalase and proteinase K) on the inhibitory property of some lactic acid bacteria against *Staphylococcus aureus* (ATCC 12600)

Organisms	Initial inhibition zone (mm)	Inhibition zone after treatment with catalase	Inhibition zone after treatment with proteinase k	SEM
Lactobacillus fermentum	13.00[a]	11.67[a]	0.67[b]	2.00
Lactobacillus acidophilus	15.00[a]	13.67[a]	0.667[b]	2.30
Lactobacillus plantarum	12.00[a]	11.33[a]	1.00[b]	1.87
Lactobacillus acidophilus	13.33[a]	12.67[a]	0.67[b]	2.07
Leuconostoc mesenteroides	9.67[a]	1.00	9.00[a]	1.45
Identity not confirmed	7.33[a]	0.33[b]	7.00[a]	1.21
Pediococcus pentosaceus	9.33[a]	0.33[b]	8.67[a]	1.51
Leuconostoc lactis	10.33[a]	0.33[b]	9.67[a]	1.67
Control	15.00[a]	13.67[a]	0.67[b]	2.30

Means in the same row with different superscripts are significantly different ($p<0.05$)
SEM= standard error of means

Table 6 Effect of different enzymes (catalase and proteinase K) on the inhibitory property of some lactic acid bacteria against *Escherichia coli* (ATCC 117755)

Organisms	Initial inhibition	Inhibition after catalase treatment	Inhibition after treatment with proteinase K	SEM
Lactobacillus fermentum	19.33[a]	18.67[a]	0.33[b]	3.13
Lactobacillus acidophilus	16.33[a]	16.00[a]	1.00[b]	2.55
Lactobacillus plantarum	15.67[a]	15.33[a]	0.67[b]	2.48
Lactobacillus acidophilus	17.67[a]	17.33[a]	0.67[b]	2.82
Leuconostoc mesenteroides	19.67[a]	0.67[b]	19.00[a]	3.14
Identity not confirmed.	10.33[a]	1.00[b]	9.33[a]	1.53
Pediococcus pentosaceus	12.33[a]	0.00[b]	12.00[a]	2.04
Leuconostoc lactis	9.67[a]	0.61[b]	9.00[a]	1.50
Control (subtilisin)	15.00[a]	13.67[a]	0.67[b]	2.30

Means in the same row with different superscripts are significantly different from one another ($p<0.05$)
SEM = standard error of means