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Full Length Research Paper

Antimicrobial properties of probiotic bacteria from various sources

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The probiotic potentials of lactic acid bacteria species isolated from various food sources (nono, ugba, ogiri, kunun-zaki and ogi) were studied. The predominant species among the isolated strains were *Lactobacillus bulgaricus, Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus brevis, Streptococcus thermophilus Leuconostoc mesenteroides* and *Pediococcus cerevisiae*. They produced bacteriocins that inhibited the growth of *Streptococcus aureus*, *Escherichia coli* and *Bacillus cereus*. *L. bulgaricus* and *L. plantarum* were selected for the fermentation of vegetable substrates-carrot, cucumber and tomatoes and cereals-rice and maize for the purpose of studying the suitability of these raw materials for probiotic juice production. The fermentations were carried out at 30°C for 72 h. The lactic acid bacteria had viability counts of 10^9 during storage at 4°C. The minimum pH attained during fermentation of samples was 3.9. The best bacteriocin preservative effect of 90% was observed in maize.

Key words: Lactic acid bacteria, tolerance, biotechnological properties, bile, ethanol.

INTRODUCTION

The lactic acid bacteria (LAB), a component of several fermented foods including dairy products have long been consumed by humans. Lactic acid bacteria are the focus of intensive research for their essential role in most fermented foods. These bacteria are able to inhibit exogenous pathogens and exert many beneficial effects on human health. Because lactic acid bacteria prohibit colonization by the invader and control the intestinal pH through the release of acetic and lactic acids, these bacteria could effectively prevent constipation and diarrhoea caused by lactose intolerance or pathogenic bacteria and also have a role in improving metabolism and lowering cholesterol level in blood (Sindhu and Khetarpaul, 2001). In recent years, consumers' demand for non-dairy-based probiotic products has increased, and probiotics have been incorporated into drinks as well as marketed as supplements in the form of tablets, capsules, and freeze–dried preparations (Shah, 2001). It has been suggested that fruit juice could serve as a good medium for cultivating probiotics (Mattila-Sandholm et al., 2002). Vegetable juices processed by lactic acid fermentation introduce a change in the beverage assortment (Karovicova et al., 2002). These juices are produced mainly from cabbage, red beet, carrot, celery and tomato. Raw material for juice production contains substances with beneficial health effect

Probiotic inhibition of pathogenic microbes in the intestinal tract may involve a variety of mechanisms, including competition for the use of nutrients, production of antimicrobial compounds, or competition for specific adhesion sites (competitive exclusion) (Gonzalez et al., 2010). Therefore, the following were the specific objectives of this study: the isolation, selection, identification and characterization of potentially probiotic strains of lactic acid bacteria from various sources in order to study

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Table 1. Antimicrobial activity (µ) of lactic acid bacteria isolated from different samples.

Lactic acid bacteria	*Escherichia coli*	*Streptococcus aureus*	*Bacillus cereus*
Streptococcus thermophilus	2	1.5	0
Lactobacillus fermentum	1.5	2	0
Lactobacillus plantarum	2	2	1
Lactobacillus brevis	2	1.5	1
Leuconostoc mesenteroides	2	1	0
Pediococcus species	2	1	0

1 µ = 5 mm zone of inhibition on culture plates.

their antimicrobial properties and to determine the optimum cultural conditions necessary for the fermentation of the substrates for the production of functional beverages.

MATERIALS AND METHODS

Bacteriocin assay

Serial dilution (10 fold dilutions) of the nono, ugba, ogiri, ogi and kunun-zaki were plated into Mann Rogosa Sharpe (MRS) agar (Oxoid, England) and then incubated for 18 h at 30°C. Selected colonies with different morphologies were subcultured on MRS agar. The subcultures were examined for bacteriocin production using a well diffusion method as described by Settanni and Corsetti (2008) using *Bacillus cereus* Ab 10 strain obtained from Nigerian Institute for Medical Research (NIMER), Yaba, Lagos, Nigeria as indicator. Wells of 5 mm were bored in the culture plates. Seven (7) species identified as lactic acid bacteria were assayed for bacteriocin production. Bacteriocin was obtained from cultures of lactic acid bacteria grown in MRS broth for 20 to 24 h using centrifugation method. Cells were harvested by centrifugation (1,000 g, 15 min) (C1500 Gallenkamp, England) and the supernatant were adjusted to different pH values (4.5, 6.0, 7.0 and 8.0) with acetate, citrate and tris buffer respectively.

Furthermore, the supernatants were sterilized using 0.2 µM pore-size filter discs (Schleicher and Schill, Dassel, Germany). These crude bacteriocin preparations were kept at 4°C until use. The effect of 2.5% ethanol, 0.1% bile, 0.1%H_2O_2 and 0.1% lactic acid were also checked on the antimicrobial activity of the bacteriocins. Inhibitory activity of the culture filtrates against *B. cereus* Ab 10, *Escherichia coli* and *Staphylococcus aureus* from NIMER, Yaba, Lagos, Nigeria were determined in duplicate by agar well diffusion assay using 65 µL of culture filtrates for each well of 5 mm diameter. Citrate buffer of pH 4.5 was added to one well as the control. Plates were incubated for 21 h at 37°C, and after an initial pre-incubation for 3 h at 15°C they were examined for zones of inhibition around the wells. The zones of inhibition were measured with a caliper in mm. One unit (µ) was arbitrarily defined as the amount (ml) of bacteriocin required to produce an inhibitory zone of 5 mm in diameter in the diffusion agar according to Settanni and Corsetti (2008).

Characterization of bacteriocin

Heat stability

A volume of 5 ml of bacteriocin in 5 test tubes was overlaid with paraffin oil to prevent evaporation and heated at 68 and 100°C with a hot plate for 10 and 20 min respectively and at 121°C for 15 min. The heat-treated bacteriocin samples were then assayed for antimicrobial activity as described above (Joshi et al., 2006).

Effect of pH

Aliquot (5 ml) of partially purified bacteriocin was taken in test tubes and the pH values of the contents were adjusted from 2 to 9 individually, using either dilute NaOH or HCl (1 M NaOH or 1 M HCl) solution. The samples were allowed to stand at room temperature for 2 h and the antimicrobial activity was assayed as described above.

Determination of preservative effect of bacteriocin

The food samples (carrot, maize, rice, tomatoes and cucumber) were sterilized and inoculated with 10^8 $cfuml^{-1}$ *B. cereus*. Initial count of inoculated samples was recorded and bacteriocin supernatant at a concentration of 0.05, 0.02, 0.1 and 0.5% were added. After 24 and 72 h, the plate count was recorded and compared with the control (without bacteriocin) using the following Equation;

$$\text{Preservative effect (\%)} = \frac{\text{Initial count}}{\text{Final count 1}} \times 100$$

Viability count during cold storage of samples

Food samples were made into paste by homogenizing them in a blender (Binatone, Japan). Samples were stored at refrigeration temperature (4°C) and the viability of the lactic acid bacteria was examined by taking 1 ml aliquot samples from the fermented juice for microbial count using the method of Wang et al. (2002) after 24, 48, 72 and 96 h incubation at 30°C, and was expressed as cfu/ml.

RESULTS AND DISCUSSION

Bacteriocin assay

The bacteriocins obtained showed antimicrobial activity against *E. coli, S. aureus* and *B. cereus*. For *E. coli*, there was 10 mm zone of inhibition activity of the bacteriocins, while for *B. cereus* there was poor bacteriocin activity or no activity against it (5 mm zone of inhibition) using the bacteriocin from *Lactobacillus plantarum* (Table 1). For

Table 2. Effect of temperature on antimicrobial activity of bacteriocins from *Lactobacillus* species.

Temperature (°C)	Time (min)	Inhibition zone diameter (mm)		
		E. coli	***S. aureus***	***Bacillus cereus***
68	10	25(100)	20(100)	21(98)
	20	23(95)	20(100)	21(98)
100	10	16(65)	15(66)	12(55)
	20	12(45)	10(44)	8(30)
121	15	0	0	0
control	-	25	20	22

Values are means of triplicate determinations (values in parentheses are in percentages).

Staphylococcus species, there was 10 mm zone of inhibition activity. This was closely followed by the bacteriocins from *Streptococcus thermophilus*, *Lactobacillus brevis*, *Lactobacillus fermentum*, *Pediococcus cerevisiae* and *Leuconostoc mesenteroides*.

Heat stability and effect of other factors on bacteriocin activity

Temperature affected the antimicrobial activity of bacteriocins from *Lactobacillus* species. Temperatures as high as 100°C for 20 min treatment led to decreased activity below 50%, while treatment of 68°C for 10 min gave 98% activity (Table 2). Moreover, the presence of 2.5% of ethanol introduced into the culture plate affected the antimicrobial activity of the lactic acid bacteria studied, while bile salt did not have significant effect on their activity. Best results were obtained when lactic acid and H_2O_2 were introduced into the culture plates signifying synergistic effect of that combination (Table 3).

Determination of preservative effect of bacteriocins on food samples

The best preservative effect of the bacteriocin from *Lactobacillus plantarum* isolated from the food samples was on maize (90%), followed by carrot juice (88%) and rice with 87% preservation using 0.5% bacteriocin concentration (Figure 1). Their preservative effects were also good on tomato and cucumber with 84 and 82%, respectively using 0.5% bacteriocin concentration (Figure 2). This showed that bacteriocin can prevent spoilage of these fermented food samples considerably.

Viability count

During the cold storage of these beverages, there was no decrease in the lactic acid bacteria count as their cfu/ml was in the range of 10^9 (Table 4). Many bacteriocin producing LAB were found in the samples. In recent years, many LAB isolated from various foods were shown to be producers of bacteriocins. Bacteriocins are proteinaceous bactericidal compounds that are produced by some microorganisms including LAB. Since a large number of these bacteriocins inhibit spoilage bacteria and food borne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus,* several authors suggested a possible application of these antibacterial compounds as biological additives in the preservation of foods (Obodai and Dodd, 2006). Most of the known bacteriocins act in a bactericidal manner, some of them resulting in cell lysis; sensitive cells are killed after exposure to the bacteriocins. Meanwhile, is known about the factors affecting the killing kinetics and the bactericidal efficiency of such bacteriocins (Leroy et al., 2002).

In this study, the bacteriocins obtained showed antimicrobial activity against *E. coli, S. aureus* and *B. cereus*. For *E. coli* there was 100% activity with > 10 mm inhibition, while for *B. cereus* there was poor activity against it with no inhibition or < 1 mm. Jones et al. (2008) discovered that the use of LAB as protective cultures in vacuum packed chill-stored meat has potential application for assuring and improving food quality, safety and market access. *Lactococcus sakei* and *Lactococcus lactis* were found to be inhibitory to *L. monocytogenes, Brochothrix thermosphacta, Campylobacter jejuni* and *Clostridium estertheticum*. These inhibitory agents appeared to be either cell associated or molecules released extracellularly with bacteriocin-like properties.

Temperature affected the antimicrobial activity of bacteriocins from *Lactobacillus* species. Temperatures as high as 100°C for 20 min treatment led to decreased activity below 50%, while treatment of 68°C for 10 min gave > 98% results. The presence of 2.5% of ethanol introduced into the culture plate affected the antimicrobial activity of the lactic acid bacteria studied, while bile salt did not have significant effect on their activity. Best results were obtained when lactic acid and H_2O_2 were introduced into the culture plates signifying synergistic

Table 3. Effect of bile, lactic acid, hydrogen peroxide, and ethanol on antimicrobial activity (µ) of lactic acid bacteria isolates on *Staphylococcus aureus* and *Escherichia coli*.

Isolate	Test organism	0.01% Bile salt	0.1% LA	0.1% H_2O_2	2.5% Ethanol
A	*Staphylococcus aureus*	2	3	3	1
	Escherichia coli	2	3	3	0
B	*Staphylococcus aureus*	2	2	3	1
	Escherichia coli	2	3	3	1
C	*Staphylococcus aureus*	2	3	2	1
	Escherichia coli	2	3	2	1
D	*Staphylococcus aureus*	2	2	3	1
	Escherichia coli	2	3	3	0
E	*Staphylococcus aureus*	2	2	3	0
	Escherichia coli	2	3	3	0
F	*Staphylococcus aureus*	2	3	3	1
	Escherichia coli	2	3	3	0
G	*Staphylococcus aureus*	2	2	3	1
	Escherichia coli	2	3	3	1

1 µ = 5mm zone of inhibition on culture plates. A, *Lactobacillus bulgaricus*; B, *Streptococcus thermophilus1*; C, *Streptococcus thermophilus2*; D, *Lactobacillus fermentum*; E, *Lactobacillus plantarum*; F, *Lactobacillus brevis*; G, *Leuconostoc mesenteroides*.

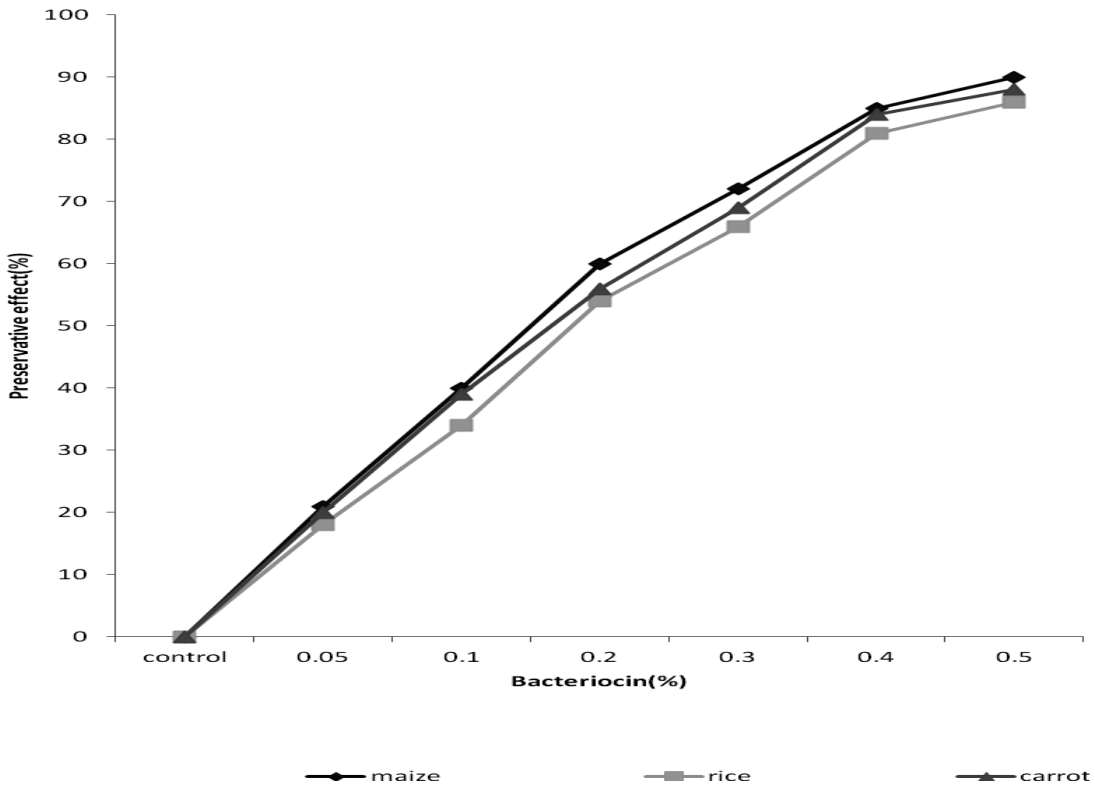

Figure 1. Preservative effect of bacteriocin of *Lactobacillus plantarum* on fermented maize, rice and carrot juice.

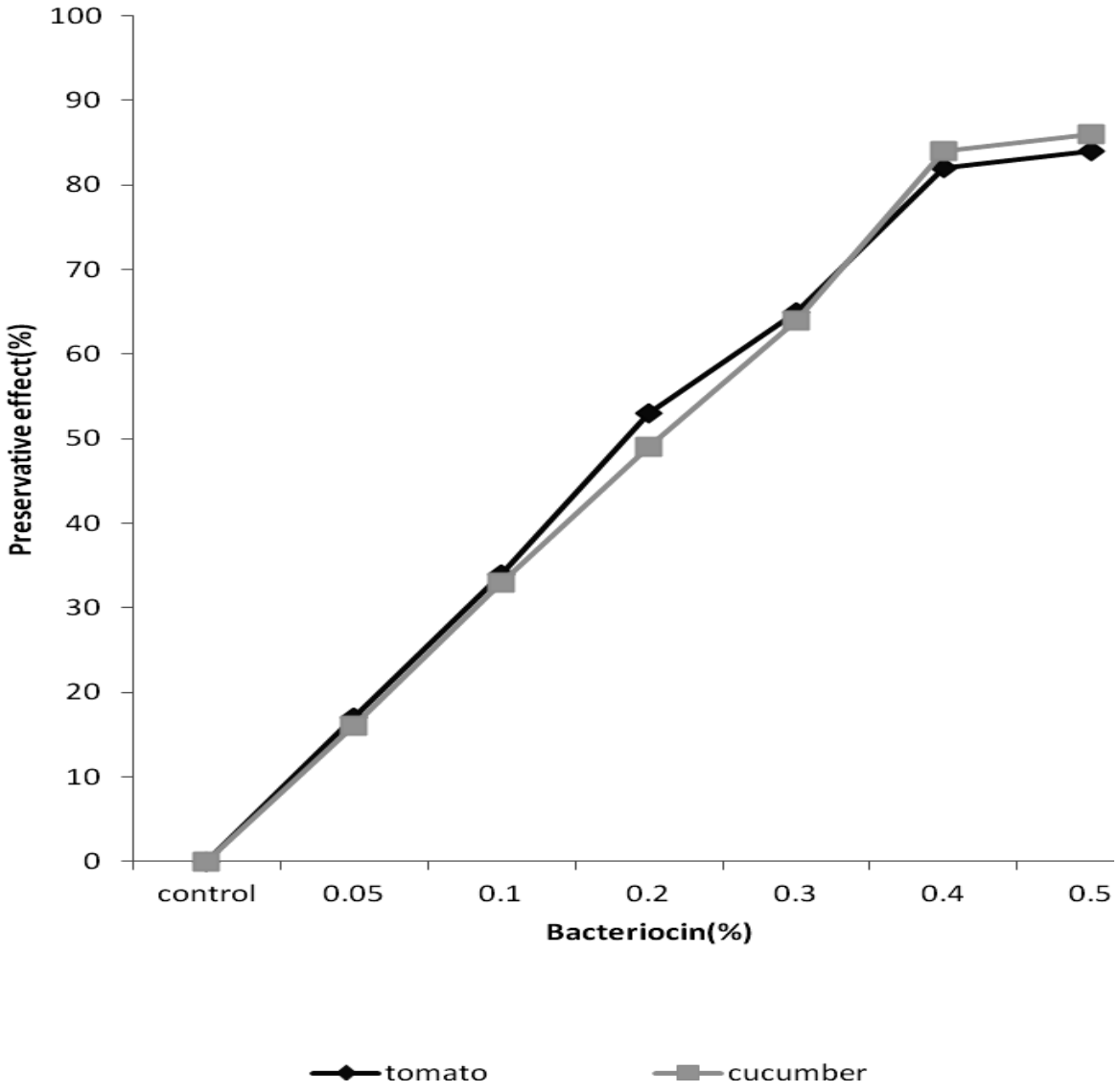

Figure 2. Preservative effect of bacteriocin of *Lactobacillus plantarum* on fermented cucumber and tomato.

Table 4. Effect of cold storage on the cell viability of lactic acid bacteria in fermented carrot, maize, rice, cucumber and tomato.

Time (h)	Survival (cfu/ml)				
	Carrot	Maize	Rice	Cucumber	Tomato
0	$1.8 \pm 0.0 \times 10^9$	$1.6 \pm 0.3 \times 10^9$	$1.3 \pm 0.2 \times 10^9$	$1.5 \pm 0.4 \times 10^9$	$2.0 \pm 0.1 \times 10^9$
24	$2.6 \pm 0.1 \times 10^9$	$3.5 \pm 0.0 \times 10^9$	$2.9 \pm 0.0 \times 10^9$	$5.8 \pm 0.1 \times 10^9$	$4.9 \pm 0.2 \times 10^9$
48	$5.4 \pm 0.2 \times 10^9$	$6.2 \pm 0.2 \times 10^9$	$8.0 \pm 0.4 \times 10^9$	$7.8 \pm 0.0 \times 10^9$	$8.7 \pm 0.3 \times 10^9$
72	$9.1 \pm 0.4 \times 10^9$	$9.3 \pm 0.3 \times 10^9$	$5.1 \pm 0.2 \times 10^9$	$9.8 \pm 0.2 \times 10^9$	$9.9 \pm 0.0 \times 10^9$
96	$7.7 \pm 0.2 \times 10^9$	$6.8 \pm 0.2 \times 10^9$	$3.1 \pm 0.1 \times 10^9$	$6.4 \pm 0.3 \times 10^9$	$6.6 \pm 0.4 \times 10^9$
120	$4.9 \pm 0.3 \times 10^9$	$4.7 \pm 0.4 \times 10^9$	$4.2 \pm 0.3 \times 10^9$	$4.8 \pm 0.2 \times 10^9$	$4.7 \pm 0.2 \times 10^9$

Values are means of triplicate determinations.

effect of that combination. Acid production is dependent upon the concentration of viable bacteria that is able to utilize carbohydrate sources available in the substrate. The ability of the LAB species to withstand bile and acid

in the *in vitro* tests showed that these organisms will be able to tolerate the acidic nature of the small intestine.

Conclusion

Probiotics could be another inexpensive substitute to the antibiotics that many microorganisms are now becoming resistant to because it is biosafe and easy in application.

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