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Antagonistic effect and bacteriocinogenic activity of Lactic Acid Bacteria isolated from *Sorghum bicolor*-based 'ogi' on food borne bacterial pathogens from cabbage

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Abstract:

Background: Lactic acid bacteria (LAB) are important organisms recognized for fermentative ability as well as health and nutritional benefits. A large number of bacteriocins from LAB have been characterized and a number of studies have indicated the potential usefulness of bacteriocin in food preservative. The objective of this study was to evaluate the antagonistic effects and bacteriocinogenic activity of LAB isolated from *Sorghum bicolor*-based 'ogi' against selected food borne bacteria from cabbage samples.
Methodology: Five samples of *Sorghum bicolor*-based 'ogi' and 5 samples of suspected infected cabbage heads were randomly collected using sterile water proof material from Abakpa main market, Abakaliki, and processed at the Applied Microbiology Laboratory of Ebonyi State University, for isolation of LAB and food borne pathogen by conventional culture and biochemical identification tests. Antagonistic effects of LAB and its bacteriocinogenic activity were determined by agar well diffusion test.
Results: Three different *Lactobacillus* species designated A, B, and C, were isolated from the *Sorghum bicolor*-based 'ogi' and 5 bacterial species were isolated from cabbage heads; *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella*, and *Shigella* species. The *Lactobacillus* species had inhibitory effect against *S. aureus*, *E. coli*, and *Shigella* species with inhibition zone diameters (IZD) of 19 mm, 10 mm, and 10 mm respectively. The crude bacteriocin extracts from the *Lactobacillus* species showed higher inhibitory activity against tested bacterial isolates at 10^{-1} (0.1ml) than at 10^{-2} dilution (0.01ml), and the inhibitory activity was higher at pH 2 than pH 6 and 7, with no activity at pH 8.
Conclusion: This study showed that LAB and its extracted bacteriocin demonstrated *in vitro* inhibitory activity against food borne pathogens isolated from cabbage heads. There is the need to further characterize the active components of the bacteriocin for possible commercial use as preservatives and potential source of new antimicrobial agent.

Keywords: Lactic acid bacteria, bacteriocin, cabbage, fermented food, 'ogi'

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Effet antagonistes et activité bactériocinogène de bactéries de l'acide lactique isolées à partir d'un «ogi» à base de sorgho bicolore sur des agents pathogènes bactériens d'origine alimentaire issus du chou

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Abstrait:

Contexte: Les bactéries de l'acide lactique (LAB) sont des organismes importants reconnus pour leur aptitude à la fermentation ainsi que pour leurs bienfaits nutritionnels et de santé. Un grand nombre de bactériocines de LAB ont été caractérisées et un certain nombre d'études ont indiqué l'utilité potentielle de la bactériocine dans un conservateur alimentaire. L'objectif de cette étude était d'évaluer les effets antagonistes et l'activité bactériocinogène du LAB isolé de «ogi» à base de *Sorghum bicolor* sur certaines bactéries d'origine alimentaire prélevées dans des échantillons de chou.
Méthodologie: Cinq échantillons d'ogi à base de *Sorghum bicolor* et 5 échantillons de têtes de choux présumées infectées ont été prélevés au hasard à l'aide d'un matériau imperméable à l'eau stérile provenant du marché principal d'Abakpa, à Abakaliki, et traités au laboratoire de microbiologie appliquée d'Ebonyi State University pour l'isolement de LAB et agent pathogène d'origine alimentaire par culture conventionnelle et tests d'identification biochimiques. Les effets antagonistes de LAB et son activité bactériocinogène ont été déterminés par un test de diffusion sur gélose.
Résultats: Trois espèces différentes de Lactobacillus désignées par A, B et C ont été isolées à partir du «ogi» à base de *Sorghum bicolor* et 5 espèces bactériennes ont été isolées à partir de têtes de chou; *Staphylococcus aureus, Escherichia coli*, espèces de *Pseudomonas aeruginosa*, *Salmonella* et *Shigella*. Les espèces de Lactobacillus avaient un effet inhibiteur contre les espèces de *S. aureus, E. coli* et *Shigella* avec des diamètres de zone d'inhibition (IZD) de 19 mm, 10 mm et 10 mm respectivement. Les extraits de bactériocine bruts de l'espèce Lactobacillus ont montré une activité inhibitrice plus élevée contre les isolats bactériens testés à 10-1 (0,1 ml) qu'à une dilution de 10-2 (0,01 ml), et l'activité inhibitrice était supérieure à pH 2 à pH 6 et à 7, sans activité à pH 8.
Conclusion: cette étude a montré que le LAB et sa bactériocine extraite ont démontré une activité inhibitrice in vitro contre les agents pathogènes d'origine alimentaire isolés de la tête du chou. Il est nécessaire de mieux caractériser les composants actifs de la bactériocine pour une utilisation commerciale éventuelle en tant que conservateurs et source potentielle de nouvel agent antimicrobien.

Mots-clés: Bactéries lactiques, bactériocine, chou, aliment fermenté, 'ogi'

Introduction:

Traditional fermented foods prepared from millet (*Pennisetum typhoideum*), sorghum (*Sorghum bicolor*) and maize (*Zea mays*) are consumed in many West African countries (1). In southern Nigeria, maize and millet are processed by fermentation into 'ogi' also known as 'akamu' and consumed commonly. 'Ogi' has been known to exhibit health promoting properties such as in the control of gastroenteritis in animals and man (1). *In vitro* and *in vivo* data have shown the probiotic, hypolipidemic, hepatoprotective and antibacterial effects of some lactic acid bacteria isolated from 'ogi' (1,2). Lactic acid bacteria (LAB) are also known for their potentials to produce antimicrobial compounds and other valuable products that inhibit growth of pathogenic microorganisms, and degrade mycotoxins (3).

In fermented foods, LAB have been known to display antimicrobial activities through production of various metabolites, including lactic acid, hydrogen peroxide, and bacteriocins (3). Many bacteriocins are active against food-borne pathogens especially *Listeria monocytogenes* (3). The predominant LAB in 'ogi' fermentation is *Lactobacillus plantarum* which is responsible for production of the main acid (lactic acid) in 'ogi' with acidity usually below pH 4. At this pH, most pathogenic microorganisms in food cannot survive, hence lactic acid fermentation has been found to reduce the growth of pathogenic microorganisms in food (4).

There are increasing interests in bacteriocins as alternative to antibiotics and chemical food preservatives. This has prompted this study with the objectives of investigating the bacteriocinogenic effects of LAB recovered from a cereal-based food, *Sorghum bicolor*, on food borne pathogenic bacteria isolated from infected cabbage.

Materials and Method:

Study setting and collection of *Sorghum bicolor*-based 'ogi' samples

Five (5) samples of *Sorghum bicolor*-based 'ogi' were randomly purchased at Abakpa market, Abakaliki metropolis, Ebonyi State during the period August and November, 2018. The samples were collected aseptically with sterile water proof materials and transported within two hours to the Laboratory of Applied Microbiology Department, Ebonyi State University, for bacteriological analysis.

Isolation/identification of LAB from Sorghum-based 'ogi' samples

The pour plate technique was used for the isolation of LAB. One gram of each 'ogi' sample was dissolved in 10 ml of water and swirled to mix properly. A tenfold serial dilution was performed from the sample homogenate by adding 1 ml to 9 ml of sterile distilled water and 1 ml aliquot of 10^{-7} and 10^{-8} of the dilution

factors were inoculated on Mann Rogosa and Sharp (MRS) agar which has been incorporated with 50µg/ml of nystatin to suppress the growth of fungi (4). The inoculated plates were incubated at 37°C for 72 hours in anaerobic jar, and suspected LAB colonies were then sub-cultured on MRS agar to obtain pure culture (5, 6).

All isolates were identified using standard microbiological techniques such as Gram staining, and biochemical tests such as citrate utilization, oxidase, indole, methyl red, voges proskauer (VP), and sugar (lactose, glucose, sucrose, fructose, maltose and mannitol) fermentation (5,6,7).

Isolation of bacterial pathogens from cabbage

Cabbage samples collected from Abakpa main market, Abakaliki, Ebonyi State were processed by first removing their outer leaves and 20g of each of the samples were weighed, washed with distilled water and blended in an electric blender. The blended samples were put in a clean beaker containing 20 ml of sterile water and sieved. The resulting filtrate was serially diluted, and 1ml of 10^{-7} and 10^{-8} dilutions were then plated on Cysteine Lactose Electrolyte Deficient (CLED) agar, Mannitol salt agar (MSA) and *Salmonella-Shigella* (SS) agar. The plates were incubated at 37°C for 24 hours. The discrete colonies of each of the bacterial isolates were identified by standard morphological and biochemical tests (5,6,7) and then sub-cultured on nutrient agar to obtain pure cultures.

Antagonistic activity of LAB

The antagonistic activity of LAB isolates was determined by the agar well diffusion technique with the cell-free supernatant of each isolate. A standardized suspension of each isolated bacterium (*E. coli*, *S. aureus*, *Ps. aeruginosa*, *Shigella* and *Salmonella* species) from the cabbage samples was prepared and inoculated onto Mueller Hinton (MH) agar plates using sterile cotton swab. The plates were allowed to dry and a sterile cork borer with a diameter of 4mm was used to cut uniform well in the agar plates. Each of the wells was filled with 0.1 ml (100 µL) aliquot of the test *Lactobacillus* isolate. The plates were then incubated at 37°C for 72 hours. Isolates exhibiting highest zone of growth inhibition were selected and screened for bacteriocin production.

Bacteriocinogenic activity of LAB

Assay of crude bacteriocin production

Bacteriocin was extracted from LAB that had highest growth inhibition by growing them first in 1 litre MRS broth and incubating for 72hours at 30°C under anaerobic conditions (8). Extract was obtained by centrifuging the culture at 12,000 rpm for 15 minutes to pellet down the cells. The pH of cell-free cultured supernatant was adjusted to 6.5 with 1M NaOH. Then, catalase (1mg/ml) was added to remove hydrogen peroxide from the supernatant (9). The supernatant was filtered through a 0.45 µm pore size membrane and the protein was precipitated using 80 % (w/v) saturated ammonium sulphate. The mixture was stirred for 1hour, after which it was stored at 4°C.

After precipitation, the mixture was centrifuged at 16,000rpm at 4°C for 30 min, and pellet was stored at 4°C. The pellet was further separated from impurities by dissolving 1ml in distilled water in an Eppendorf tube and centrifuging at 10,000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet containing bacteriocin was washed with deionized water, dispensed into another Eppendorf tubes, and centrifuged once again at 10,000 rpm for 15 minutes at 4°C to pellet down the proteins. After discarding the supernatant, the pellet was dissolved in 500 µl of 0.1M sodium phosphate buffer (pH 7.0) and the total volume was made up to 2 ml.

The sample was then loaded in a pre-treated dialysis tubing cellulose membrane (18-20 length) and dialyzed in a 3 litre 0.1M sodium phosphate buffer (pH 7.0) for 2 hours, following which the buffer was changed and the sample further dialyzed overnight at 4°C. After 24 hours dialysis, the sample was reloaded in Eppendorf tubes and centrifuged at 10,000 rpm at 4°C for 30 minutes and then stored at -20 °C.

Partial precipitation of proteins from crude bacteriocin

The crude bacteriocin samples were dispensed into Eppendorf tubes and centrifuged at 12,000 rpm for 15 mins at 4°C to pellet down the cells. The supernatant was poured into a 50 ml capacity beaker in an ice pack at a temperature of 4°C and 15g of ammonium sulphate [$(NH_4)_2SO_4$] in dry form was measured and dissolved in the supernatant. The mixture was stirred for 1 hour and stored at 4°C for 24 hours. The stirring was carefully done to prevent foaming that may lead to protein denaturation (10).

Determination of bacteriocin activity

The agar well diffusion assay was used to determine the bacteriocin activity of the LAB isolates (11). Ten ml of partially purified bacteriocin was serially diluted up to 10^{-2} using saline diluent. An overnight culture of bacteria

isolated from cabbage grown in Tryptic soy broth (TSB) at 37°C was diluted in saline to a 0.5 McFarland standards. The suspension was inoculated on MH agar plates using a sterile cotton swab. The plates were allowed to dry and a sterile cork borer with a diameter of 4mm was used to cut uniform circular wells in the agar plates. Each of the wells was filled with 0.1ml (100µL) aliquot of the partially purified bacteriocin from the LAB. The plates were kept at 4°C for 2hours to ensure diffusion of the supernatant fluid into the agar, and then incubated at 37°C for 24 hours. With the aid of meter rule, the antimicrobial activity was determined by measuring the diameter of zones of inhibition around the wells.

Effect of pH on crude bacteriocin activity

A 5ml aliquot of crude bacteriocin from the LAB was distributed into different test tubes. To each of the respective test tubes, 1 ml of sodium hydroxide (NaOH) and hydrogen chloride (HCl) was added to obtain pH values of 2, 3, 4, 5, 6, 7, and 8 which was confirmed with the aid of calibrated Jenway pH meter. The solutions were allowed to stand at room temperature for 2 hours. Aliquots of 50 µl from each test tube were placed in wells (4mm diameter) of MH agar plates that have been inoculated with overnight broth cultures of the isolated bacteria (*S. aureus*, *E. coli*, *Ps. aeruginosa, Salmonella*, and *Shigella* species) from cabbage. These were incubated at 30°C for 24hours, following which the zones of inhibition around the wells were measured in mm with a meter rule.

Results:

Morphological and biochemical characteristics of LAB

Table 1 shows the morphological and biochemical characteristics of LAB isolated from samples of *Sorghum bicolor*-based 'ogi' collected from Abakpa main market, Abakaliki, Ebonyi State. Three different LAB groups; *Lactobacillus* species A*, Lactobacillus* species B, and *Lactobacillus* species C were identified. Table 2 shows that *S. aureus*, *E. coli*, *Ps. aeruginosa*, *Salmonella* and *Shigella* species were isolated from cabbage samples collected from the same market.

Table 1: Morphological, microscopic and biochemical characteristics of the Lactic Acid Bacteria isolated from Sorghum-based 'ogi'

Morphological Characteristics		Microscopic Characterization		Biochemical Tests Sugar Fermentation										
Shape	Colour	Gram reaction	Motility test	Catalase Test	Mannose	Arabinose	Lactose	Ribose	Glucose	Mannitol	Sucrose	Fructose	Maltose	Suspected LAB
Rod	White	+	-	-	+	-	+	+	-	+	+	-	+	*Lactobacillus* species A
Rod	Cream	+	-	-	-	-	+	+	+	-	+	+	+	*Lactobacillus* species B
Rod	Dirty white	+	-	-	-	+	+	+	+	+	+	-	-	*Lactobacillus* species C

+ = positive - = negative

Tables 2: Morphological, microscopic and biochemical characteristics of bacteria isolated from cabbage sample

Morphological Characteristics		Biochemical Tests						Sugar Fermentation Test							
Shape	Colour	Gram reaction	Motility test	Citrate Test	Oxidase Test	Coagulase Test	Indole Test	Lactose	Glucose	Sucrose	Catalase Test	Urease Test	Voges Proskauer	Methyl Red	Probable Organism
Cocci	Yellow on mannitol salt agar	+	-	-	-	+	-	+	+	-	+	+	+	+	*Staphylococcus aureus*
Rods	Opaque yellow on CLED	-	+	-	-	-	+	+	+	+	+	-	-	+	*Escherichia coli*
Rods	Greenish on CLED	-	+	-	+	-	-	+	+	+	+	-	-	+	*Pseudomonas aeruginosa*
Rod	Pink on SS agar	-	-	-	-	-	-	-	+	-	+	-	-	+	*Shigella* species
Rods	Black on SS agar	-	+	-	-	+	-	-	+	-	+	-	-	+	*Salmonella* species

+ = positive; - = negative

Antagonistic effects of isolated LAB

Lactobacillus species group A produced inhibitory zone diameters of 19, 10, 10, 12 and 14mm against *S. aureus, E. coli, Ps. aeruginosa, Salmonella,* and *Shigella* species respectively (Fig1). *Lactobacillus* species group B produced inhibitory zone diameters of 16, 10, 12, 14 and 10mm against *S. aureus, E. coli, Ps. aeruginosa, Salmonella,* and *Shigella* species respectively. *Lactobacillus* species group C produced inhibitory zone diameters of 14, 14, 12, 12 and 10mm against *S. aureus, E. coli, Ps. aeruginosa, Salmonella*, and *Shigella* species respectively.

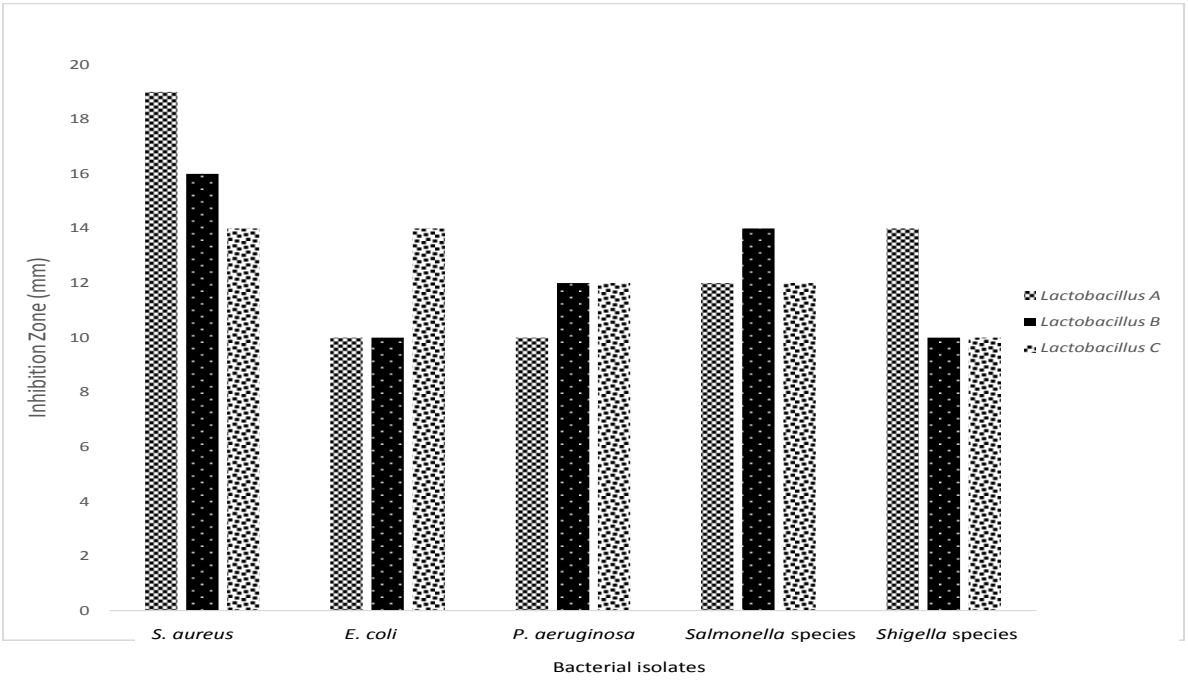

Fig 1: The Inhibition Zone Diameter (mm) of LAB isolated from *Sorghum bicolor*-based 'ogi' against food borne bacterial isolates from cabbage

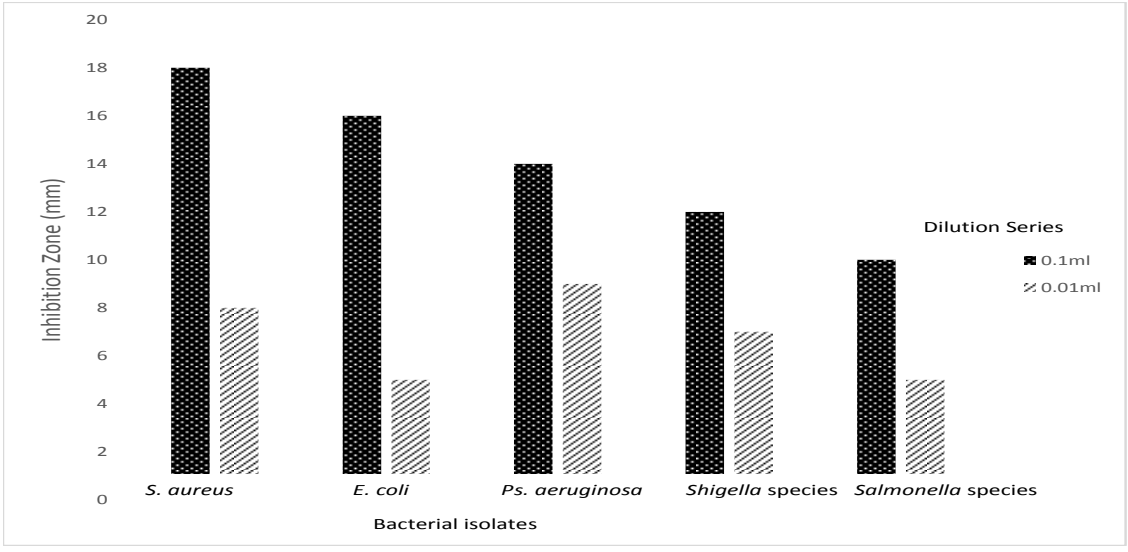

Fig 2: Antimicrobial activity of diluted crude bacteriocin from *Lactobacillus* species A i solated from *Sorghum bicolor*-based 'ogi' against bacterial isolates from cabbage samples

Bacteriocinogenic activity of LAB

The antimicrobial activity of diluted crude bacteriocin from *Lactobacillus* species A as depicted in Fig 2 showed that 10^{-1} (0.1ml) dilution produced inhibition zone diameters of 18, 16, 14, 12, and 10mm against *S. aureus, E. coli, Ps. aeruginosa, Shigella* species, and *Salmonella* species respectively, while 10^{-2} (0.01ml) dilution produced inhibition zone diameters of 8, 5, 9, 7, and 5mm against *S. aureus, E. coli, Ps. aeruginosa, Shigella* and *Salmonella* species respectively.

The antimicrobial activity of the crude bacteriocin from *Lactobacillus* species group B showed that 10^{-1} dilution produced inhibition zone diameters of 16, 11, 13, 10, and 15mm against *S. aureus, E. coli, Ps. aeruginosa, Shigella*, and *Salmonella* species respectively, while 10^{-2} dilution produced inhibition zone diameters of 7, 4, 5, 8, and 11mm against *S. aureus, E. coli, Ps. aeruginosa, Shigella,* and *Salmonella* species respectively (Fig 3).

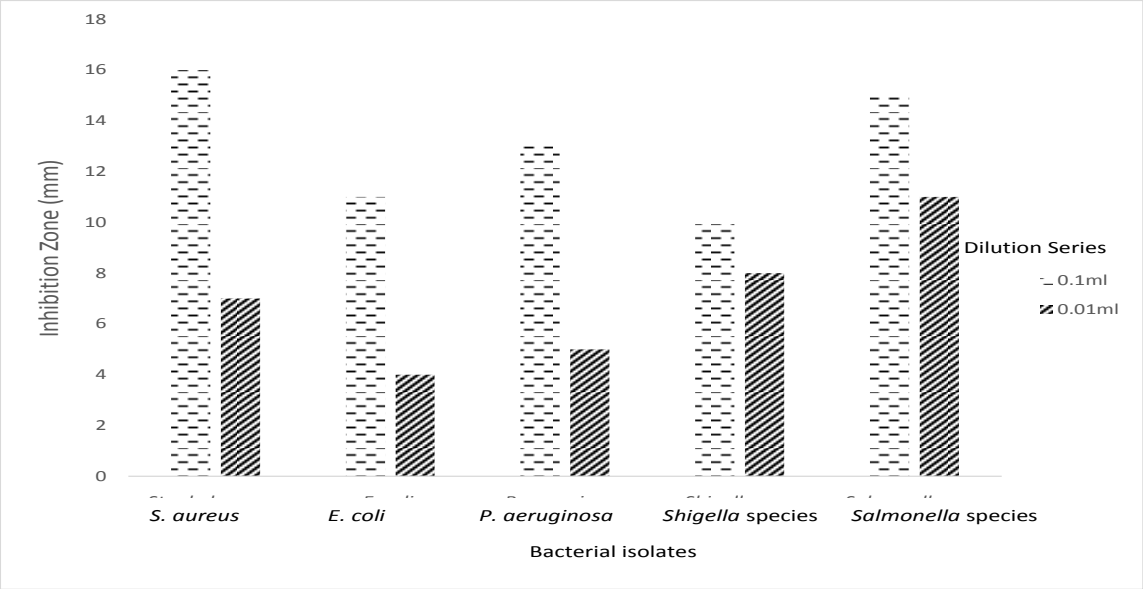

Fig 3: Antimicrobial activity of diluted crude bacteriocin from *Lactobacillus* species B from *Sorghum bicolor*-based 'ogi' against bacterial isolates from cabbage samples

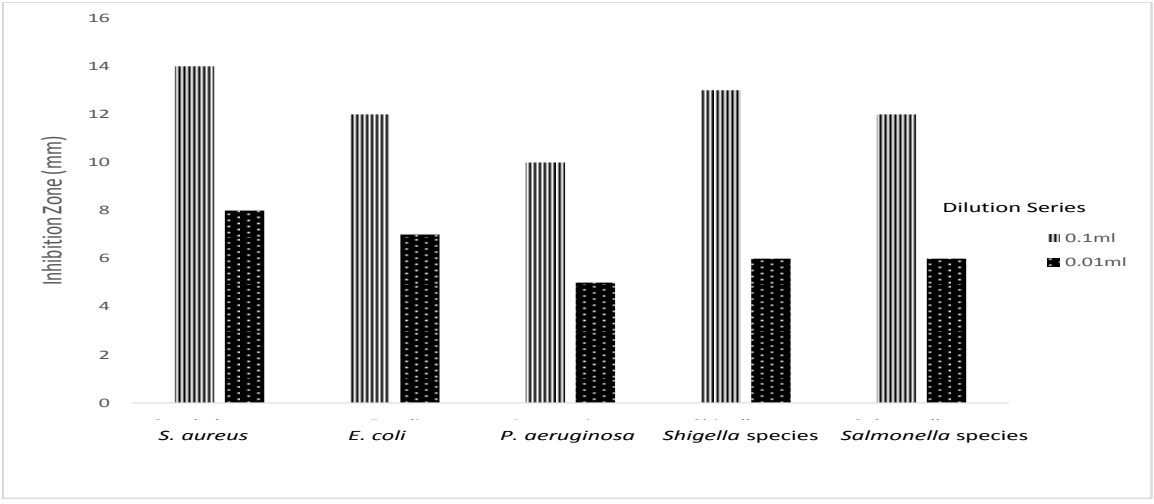

Fig 4: Antimicrobial activity of diluted crude bacteriocin from *Lactobacillus* species C isolated from *Sorghum bicolor*-based 'ogi' against bacterial isolates from cabbage samples

Table 3: Effect of pH on antimicrobial activity of bacteriocin against bacteria isolated from *Sorghum bicolor*-based 'ogi'

pH	Isolates zone of inhibition (mm)				
	S. aureus	*E. coli*	*Ps. aeruginosa*	*Salmonella* species	*Shigella* species
2	21	17	14	19	18
3	14	13	11	12	11
4	13.5	10	9	10	11
5	11	11	10	9	10
6	9	7	4	5	4
7	5	3	5	2	3
8	3	1	4	NI	1

NI = no inhibition

The antimicrobial activity of the crude bacteriocin from *Lactobacillus* species group C depicted in Fig 4 showed that 10^{-1} dilution produced inhibition zone diameters of 14, 12, 10, 13, and 12mm against *S. aureus, E. coli, Ps. aeruginosa, Shigella*, and *Salmonella* species respectively, while 10^{-2} dilution produced inhibition zones of 8, 7, 5, 6, and 6mm for *S. aureus, E. coli, Ps. aeruginosa, Shigella*, and *Salmonella* species respectively (Fig 4).

Effect of pH on stability of bacteriocin

Table 3 showed that at pH of 2, 3, 4, 5, 6, 7 and 8, inhibition zone diameters (IZDs) of 21, 14, 13.5, 11, 9, 5 and 3mm respectively were recorded against *S. aureus;* IZDs of 17, 13, 10, 11, 7, 3 and 1mm against *E. coli;* IZDs of 14, 11, 9, 10, 4, 2, and 0mm against *Ps. aeruginosa*; IZDs of 19, 12, 10, 9, 5, 2, and 0mm against *Salmonella* species and IZDs of 18, 11, 11, 10, 4, 3, and 1mm against *Shigella* species.

Discussion:

Lactic acid bacteria satisfy the increasing demand from consumers for food that contain lower concentration of chemical preservatives, as bacteriocin are natural antimicrobials, produced by bacteria normally present in fermented food. The present study sought to determine the effect of partially purified bacteriocin on some indicator organisms that are known to cause food spoilage. In this study, *Lactobacillus* species were isolated from five samples of *Sorghum bicolor*-based 'ogi' which Ohenhen *et al*., had previously isolated from fermented 'ogi' samples in their study (12). *Lactobacillus* species have also been reported to be predominant in fermented foods (13).

Staphylococcus aureus, *E. coli*, *Ps. aeruginosa*, *Shigella*, and *Salmonella* species were also isolated from infected cabbage collected from Abakpa main market, which is similar to the study of Sujeet and Vipin (14), who reported the presence of the same bacterial species in cabbage and other salad vegetables. These bacteria have been implicated as common food borne infectious pathogens (15). It is well established that food is a valuable source of nutrients for certain microbes, and their growth on the food may result in unpleasant smell, bad taste, and poor appearance of food (16).

The isolated LAB from *Sorghum bicolor* showed antagonistic activity against the food borne bacteria isolated from cabbage. It was observed that all the *Lactobacillus* species and their purified bacteriocins (at the two dilutions) produced highest IZDs against *S. aureus* and lowest against *E. coli*. This implies that they possess higher activity against Gram positive than Gram negative bacteria. Our finding is in agreement with that of Rammelsberg *et al*. (17), who observed that antimicrobial activity of purified bacteriocin extracted from *L. parecasei* subsp *tolerans* was more active against *S. aureus* and *Listeria monocytogenes* than *E. coli*. The cell wall of Gram positive bacteria is made of large amount of peptidoglycan which constitutes about 90% of the dry cell wall mass (18), and which may be the site of action of the bacteriocin. In contrast, the more complex cell wall composition of Gram negative bacteria made them resistant to many antimicrobial compounds especially those with high molecular size that may be unable to penetrate the cell wall to reach their possible sites of inhibitory action on the bacteria (15). Our observation however contrast that of Ohenhen *et al*., (12), who observed highest and lowest zone of inhibitions by *Lactobacillus plantarum* for *E. coli* and *S. aureus* respectively.

The actions of the bacteriocin on *S. aureus* showed dose-dependent inhibitory effects with larger IZDs at 10^{-1} dilution (higher concentration) compared to 10^{-2} dilution (lower concentration), which agrees with the report of Ohenhen *et al*., (12), who observed similarly that 10^{-1} dilution produced higher inhibitory activity against indicator bacteria than 10^{-2}

dilution. Because bacteriocins do not act equally against target species, many researchers have examined the affinity of bacteriocin to specific species and strains (19). The crude bacteriocin extract in our study demonstrated higher antimicrobial activity at pH 2 compared to pH 6 and 7, and no activity was demonstrated at pH 8. This could be due to the fact that the producer organism (*Lactobacillus* species) has a high tolerance for low pH. This observation is similar to that of Ogunbanwo *et al*., (8), who reported that purified bacteriocin extract recovered from *L. plantarum* was more active at pH 2 and 6, than at pH 10 and 12.

Conclusion:

Our study showed that LAB isolated from *Sorghum bicolor*-based 'ogi' and partially purified bacteriocins from them demonstrated inhibitory activity maximal at pH 2 against Gram positive *(S. aureus*) and Gram-negative bacteria (*E. coli, Ps. aeruginosa, Salmonella*, and *Shigella* species). The LAB have potential for use as safer bio-preservatives in acidic food products in preference to chemical preservatives. This study indicates that partially purified bacteriocins have the potential to replace chemical preservatives in food products. Additionally, the bacteriocin produced by the LAB isolates in this study were noted to have maximum activity at acidic pH 2-6, which supports their use as bio-preservatives in acidic food products such as fruits juices, in preference to chemical preservatives that may have adverse effects on human body system.

Conflict of interest:

Authors declare no conflicts of interest

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