

Antibacterial activity of *Lactobacillus* species isolated from raw goat milk against *Staphylococcus aureus*

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Abstract

Lactobacillus spp. are known to have many health benefits and are mostly used as probiotics. They can cause inhibition of certain micro-organisms by the reduction of pH due to production of lactic acid, hydrogen peroxide and antimicrobial compounds such as bacteriocins. The antibacterial activity of *Lactobacillus* spp. Against *Staphylococcus aureus* was evaluated using agar well diffusion method. Three raw goat milk samples were pooled together, serially diluted, cultured on MRS (De Man, Rogosa, Sharpe) Agar, and purified. Four *Lactobacillus* species were isolated and identified by biochemical tests as *L. lactis*, *L. plantarum*, *L. Casei* and *L. Fermentum*. Antibacterial activity of the four isolates against the test organism, *Staphylococcus aureus*, was determined by agar well diffusion method following the extraction of crude and precipitated bacteriocin from the isolates. All the isolates produced antagonistic effects against the test organism with maximum zones of growth inhibition produced by precipitated bacteriocin [14.0mm (*L. casei*) to 16.0mm (*L. lactis*)] whereas minimum zones of growth inhibition was produced by crude bacteriocin [8.0mm (*L. casei*) to 11.0mm (*L. lactis*)]. The results obtained confirmed that the four *Lactobacilli* isolated have growth inhibitory effects against *Staphylococcus aureus* and can therefore serve as important probiotic organism. It also goes further to show the importance of *Lactobacillus* bacteriocins as food bio-preservatives. This therefore justifies an in-depth investigation for the identification and application of these organisms in generating alternative antibacterial therapies for human and animal diseases to counter the problem of bacteria antibiotic resistance in recent years.

Keywords: *Lactobacillus*; *Staphylococcus*; Antibacterial; Resistance; Bacteriocin; Medicinal

1. Introduction

Lactic Acid Bacteria (LAB) constitutes a group of genus that has the following common features: cocci, rods and a basic composition of DNA below 50 mol % G+C. They are typically Gram positive, mesophilic, can grow within 5°C to 45°C under aerobic, anaerobic or microaerobic conditions and are asporogenous. In addition, they are oxidase and catalase negative, cannot reduce nitrate to nitrite and are incapable of producing indole or hydrogen sulphide. This group consists of numerous genera: *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Carnobacterium*, *Streptococcus*, *Enterococcus*, *Aerococcus*, *Bifidobacterium* and *Pediococcus* [10, 6, and 14].

Lactic Acid Bacteria (LAB) are found in a plethora of niches, including plant material, fermented dairy, vegetable and meat products, and sour dough breads. Lactic acid bacteria have been long established as the normal flora in fermented food [12].

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In recent years LAB have been playing important role in the food and feed fermentation and preservation either as the natural microflora or as starter culture added under controlled conditions. Foods fermented by LAB are rendered more adapted for longer preservation and have improved textures, flavours and tastes [15]. This is due to the fact that they have been recognized as GRAS (Generally Recognized as Safe) microorganisms. *S. thermophilus* is a lactic acid bacterium of major importance in food industry e.g. the manufacture of yogurt. The preservative effect exerted by LAB is mainly due to the production of organic acids (such as lactic acid) [11].

Lactic acid producing bacteria are known to have other various beneficial functions such as anti-tumour activity, reduction of serum cholesterol, alleviation of lactose intolerance, stimulation of the immune system, enhancement of resistance against certain bacteria (antimicrobial action) and prevention of diarrhea's [34, 2]. Based on these functions, different kinds of LAB have been developed as probiotics, and the market volume of probiotics has rapidly increased [20, 8].

The antimicrobial effect of LAB has been appreciated by man for more than 1000 years ago and has enabled him to extend the shelf life of many foods through fermentation processes since it can inhibit the growth of such microorganisms including spoilage and pathogenic bacteria [5, 29].

The antibacterial action of lactic acid is largely, but not totally, assigned to its ability in the non-dissociated form to penetrate the cytoplasmic membrane of organisms, resulting in reduced intracellular pH and disruption of the Trans membrane proton motive force [33].

This effect may also have resulted from the action of organic acids, hydrogen peroxide, diacetyl and/or a compound(s) called bacteriocin(s) [30, 36, and 19]. The inhibition of pathogenic bacteria such as *Staphylococcus aureus* by lactic microflora was announced by Heikkila and Saris [21].

Bacteriocins are proteinaceous compounds that mainly inhibit closely related species [22]. Some bacteriocins have been shown to possess the ability to inhibit the actions of unrelated genera such as *Clostridia*, *Listeria*, enter pathogenic bacteria and gram-negative bacteria.

An arid variety of LAB, that produce antimicrobial substance such as hydrogen peroxide, CO₂, diacetyl, acetaldehyde, D-isomers of amino acids, reuterin and bacteriocins have already been isolated from many fermented products. This includes naturally fermented sausages [5] fermented milk and dairy products and silage which is stored fermented vegetable product [27].

The discovery of bacteriocins gave a new way for food development in better hygienic quality [7]. In recent years, there have been many reports on bacteriocins that are produced by lactic acid bacteria. However, most reports deal with bacteriocins that are produced by various *Lactococci*, *Pediococci*, *Leuconostoc*, *Enterococci* and *Lactobacilli* [26]. The search for new strains of lactic acid bacteria that produce antimicrobial substances is a universal objective for the creation of new cultures starter with a high biosafety for fermented food.

In order to avoid the side effect of chemical preservatives these last years, the bacteriocins or strains of lactic acid bacteria for applications as bio-preservative caused many research tasks [3]. Several lactic acid bacteria bacteriocins offer potential applications in food preservation and the use of bacteriocins in the food industry can help reduce the addition of chemical preservatives as well as the intensity of heat treatments, resulting in foods which are more naturally preserved and richer in organoleptic and nutritional properties [18].

Food contamination by *Staphylococcus aureus* is a major problem to consumer's health in Nigeria, especially during the summer period. The pathogens come in contact with the food during both preparation and preservation. Improper food preparation includes; cooking with water polluted by faecal materials, improperly washed vegetables, fruits and especially raw meat and fish with blood. Some of the pathogens die, while cooking, whereas some persist and cause diseases in the enteric and other parts of the body. Moreover, cooking for a short time also lets the pathogens to grow and thrive in food products. Foods in restaurants and particularly food from street side shops cause the most cases of food borne diseases [32].

When it comes to the spread of pathogens by ill preservation, it involves storing the food at wrong temperature, storing both raw and cooked food together, improper pasteurization of milk, not storing food in proper temperature and etc. In fact, dairy products and meat products are the most common sources for spreading diseases.

In recent years also, bacterial antibiotic resistance has been considered a problem due to the extensive use of classical antibiotics in treatment of human and animal diseases [38, 24, and 35]. As a consequence, multiple resistant strains have appeared and have spread causing difficulties and the use of antibiotics as growth promoters has been restricted. Therefore, the continued development of new classes of anti-microbial agents has become of increasing importance to medicine [23, 17]. Application of some bacterial peptides as anti-microbial substances can be one plausible alternative for abusive use of antibiotics in food and feed products.

Lactic acid produced by lactic acid starter culture bacteria functions as a natural antimicrobial component, having a generally recognized as safe status. Lactic acid is able to inhibit the growth of many types of food spoilage bacteria, including gram-negative species of the families *Enterobacteriaceae* and *Pseudomonadaceae* [13]. In this study Lactic acid bacteria (*Lactobacillus*) were isolated from raw goat milk and screened for antimicrobial activity against *Staphylococcus aureus*. This study was aimed at screening the antimicrobial activities of *Lactobacillus* isolated from raw goat milk against *Staphylococcus aureus*.

2. Material and methods

2.1. Sample Collection

A total 30 mL of raw, fresh goat milk sample was collected in sterile container from three different goats of different breeds. Ten (10) mL was collected from each and the samples were transported immediately to the laboratory in ice packs. In the laboratory, the three raw goat milk samples were bulked together prior to isolation.

2.2. Microbial Isolation

From the bulked milk samples, 1 mL was collected using sterile pipette and diluted in 9mL of sterile distilled water to make an initial tenfold dilution (10^{-1}). The solution was diluted serially in distilled water up to the 3rd fold and 1 mL of each sample (dilution) was cultured on sterile MRS Agar plates in duplicates by pour-plate method. The preparation was incubated in microaerophilic condition at 37°C for 48 hours. Viable colonies were calculated and expressed as Log₁₀CFU/mL. The Total Viable counts were determined using the formula below;

$$\text{TVC (CFU/mL)} = \frac{1}{V} \times N \times D$$

Where, V = Volume of inoculums, N = Number of colonies counted and D = Dilution factor

Pure colonies were obtained by sub-culturing isolates on new MRS Agar for 24 hours and in slant agars. The pure isolates were subjected to identification as per the Bergey's Manual of Determinative bacteriology using relevant microscopic and biochemical tests. The morphological characters of the isolates like colour, shape, margin and surface and cell morphology such as shape, arrangement, Gram and spore staining reaction, as well as motility were studied and reported.

2.3. Identifications

Furthermore, biochemical tests were used for the identification of bacterial isolates and the results were also recorded.

2.4. Extraction of Bacteriocin

After identification, cultures from each isolate were selected and inoculated into MRS broth and incubated at 37°C for 24–48 hours under microaerophilic condition. After fermentation, broth was centrifuged at 12000 rpm for 15 min. and the cell free supernatant (CFS) was collected. The pH of CFS was adjusted to 7.0 (neutral) with 2N NaOH and labelled as crude bacteriocin preparation [37].

Since some bacteria secrete bacteriocins in minute amounts, there is need for purification of the cell free supernatant (crude bacteriocin) by the method of Lowy, [25]. For this, ammonium sulphate was added to the CFS till 70% saturation and then the solutions were incubated at 4°C for 30 minutes with mild shaking. After precipitation, the broth was centrifuged at 15,000 rpm for 15 minutes and the supernatants were decanted until only 1mL of CFS remained in the test tube. Precipitate was collected and stored in 0.2M Sodium phosphate buffer (pH 6.9) and preparation [28].

2.5. Antibacterial activity testing

With the help of agar well diffusion method of Park *et al.*, [31] antimicrobial activity of both the crude and precipitated bacteriocin preparations were checked for to confirm the presence of antimicrobial substances in it.

The antibacterial activity of the isolates of *Lactobacillus* against the test organism, *Staphylococcus aureus*, was determined by the agar well diffusion method [31].

Prior to the antimicrobial activity testing, Muller-Hilton (MH) agar plates were prepared according to manufacturer's instruction and the test organism was inoculated and incubated overnight in nutrient agar (NA). After 24 hours, discrete bacterial colonies from the NA plates were picked and transferred to sterile saline solution. Using a sterilized cotton swab, Muller-Hilton (MH) agar plates were spread with the test organism's saline suspension and within 15 minutes, wells of 5 mm were cut using a sterilized cork borer.

2.6. Statistical Analysis

Data from this study were entered and analyzed using Statistical Package for Social Sciences (SPSS) version 21 and Microsoft Excel.

3. Results

Table 1 shows the viable bacteria count from each of the samples from 10^{-1} to 10^{-3} dilutions after 48 hours incubation. From the table, the bacterial load of the bulked milk sample decreases with increased dilution of the sample. The mean viable count was obtained mathematically;

$$\text{mean viable count} = \frac{\text{Plate 1} + \text{Plate 2}}{2}$$

Table 1 Viable bacterial count from each of the samples from 10^{-1} to 10^{-3} dilutions after 48hours incubation

Dilution factor	Viable count		Mean Viable Count	CFU/mL	Log ₁₀ CFU/mL
	Plate 1	Plate 2			
10^{-1}	32	28	30	3.0×10^2	2.43
10^{-2}	23	24	23.5	2.35×10^3	3.37
10^{-3}	16	18	17	1.7×10^4	4.23

Table 2 shows the colonial morphologies and microscopic appearances of the bacterial isolates. All isolates showed positive Gram stain reaction, but spore stain negative. Isolate 2, 3 and 4 appeared rod shaped, whereas isolate 1 was coccobacilli in form. However, all isolates were non-motile.

Table 2 Colonial and cellular morphologies of pure bacterial isolates

Isolate Number	Colony Morphology	Gram Reaction	Shape	Motility	Spore
1	circular cream-white smooth colonies	+	Cocobacilli	-	-
2	White slightly raised smooth round colonies	+	Rod	-	-
3	White circular smoothraised colonies	+	Rod	-	-
4	Small cream-white smooth round colonies	+	Rod	-	-

*(-) = Negative; *(+) = Positive

Table 3 shows relevant biochemical tests performed on the bacterial isolates. The isolates were identified as *L. lactis*, *L. plantarum*, *L. Casei* and *L. Fermentum* by their respective biochemical test reactions.

Table 3 Biochemical identification of the bacterial isolates

Isolate Number	Carbohydrate utilization				Growth at 45°C	Indole	Nitrate	Methyl red	Citrate	Voges Proskauer	Oxidase	Catalase	Isolate Identity
	Dextrose	Lactose	Fructose	Maltose									
Isolate 1	A	A	A	A	+	-	-	-	-	-	-	-	<i>Lactobacillus lactis</i>
Isolate 2	A	A	A	NA	-	-	-	-	-	-	-	-	<i>Lactobacillus plantarum</i>
Isolate 3	A	A	A	A	+	-	-	-	-	-	-	-	<i>Lactobacillus casei</i>
Isolate 4	A	A	A	A	-	-	-	-	-	-	-	-	<i>Lactobacillus fermentum</i>

(-) = Negative, (+) = Positive, (NA) = No Acid production, (A) = Acid production

Table 4 shows the results of the antimicrobial susceptibility testing for bacterial isolates measured in mm. However, it seems that the antimicrobial activity of the isolates have no direct correlation with extent of antimicrobial activity exhibited as little or no difference is observed in the zones of inhibition for both the crude and precipitated bacteriocin.

Table 4 Antimicrobial susceptibility testing for bacterial isolates

Organisms	Zone of Inhibition (mm)		Mean zone of inhibition (mm)	Mean \pm SD zone of inhibition
	Crude extract	Precipitated (Purified)		
<i>L. lactis</i>	10.0	16.0	13.0	13.0 \pm 4.24
<i>L. plantarum</i>	11.0	15.0	13.0	13.0 \pm 2.83
<i>L. Casei</i>	8.0	14.0	11.0	11.0 \pm 4.95
<i>L. Fermentum</i>	11.0	15.0	13.0	13.0 \pm 2.12

4. Discussion

Lactobacillus spp. are known to have many health benefits and are mostly used as probiotics. They can cause inhibition of certain micro-organisms by the reduction of pH due to lactic acid production, hydrogen peroxide production and production of antimicrobial compounds such as bacteriocin.

In recent years, bacterial antibiotic resistance has been considered a problem due to the extensive use of classical antibiotics in treatment of human and animal diseases [35] and hence the continued development of new classes of antimicrobial agents has become of increasing importance to medicine [17, 23].

In this study, aimed at assessing the antibacterial potency of *Lactobacilli* isolates obtained from raw goat milk samples was embarked on and four *Lactobacillus* species identified were tested for their antibacterial activity against *Staphylococcus aureus*.

These isolates have also been reported as the most predominant species of *Lactobacillus* in raw goat milk in a similar study (Characterization of *Lactobacillus* from Algerian Goat's milk based on phenotypic, 16S rDNA Sequencing and their

technological properties) conducted by Ahmed *et al.* [1]. The findings of this study therefore agree with Ahmed *et al.*, report but for the substitution of *L. fermentum* with *L. paracasei*.

All the isolates in this study produced antagonistic effects against the test organism *Staphylococcus aureus*. These results support the reports of Arokiyarny *et al.* [4] who isolated the bacteriocin producing *Lactobacillus* species from traditional milk products and reported its antimicrobial activity against common pathogens.

In this study, it was also observed that the maximum zones of growth inhibition against test organism was produced by precipitated (purified) bacteriocin with diameter ranging from 14.0mm (*L. casei*) to 16.0mm (*L. lactis*), whereas minimum zones of growth inhibition was produced by crude bacteriocin with diameter ranging from 8.0mm (*L. casei*) to 11.0mm (*L. lactis*), which corroborate the reports of Enan *et al.* [16]. The difference in antibacterial activity of the purified (precipitated) and crude extracts on the isolates, which showed inhibition zones between 0.5 to 13.00 mm in diameter by crude bacteriocin and zones of growth inhibition between 12 to 32 mm for partially purified (precipitated) bacteriocin is traceable to the purity level of the precipitated bacteriocin used compared to the crude bacteriocin.

The results of present study showed that the four different *Lactobacillus* spp. isolated were important probiotic organisms which have growth inhibitory effects against *Staphylococcus aureus*.

In this study also, it was discovered that all isolates have antibacterial effects against *Staphylococcus aureus* which is a Gram-positive bacterium. This is in line with the report of Savadogo *et al.* [35] who confirmed that gram-positive bacteria are more sensitive than gram-negative. Jamalifar *et al.*, researched on screening of *Lactobacillus* strains against *Pseudomonas aeruginosa* and they found that some *Lactobacilli* such as *Lactobacillus acidophilus* showed significant inhibitory activity against the multidrug resistant clinical isolates of *Pseudomonas aeruginosa*.

5. Conclusion

The results obtained in this study confirmed that *Lactobacilli* possess growth inhibitory effects against *Staphylococcus aureus* and can therefore serve as important probiotic organism justifying a more in depth investigation for their identification and application as food bio preservatives. It also showed the health importance of lactobacillus which is hinged on the unique property they possess and which can be harnessed in generating alternative antibacterial therapies for human and animal diseases to counter the problem of bacteria antibiotic resistance.

However, further work is needed to ascertain whether there is a relationship between the species of *Lactobacillus* isolated from raw goat milk samples and their antibacterial potential. Also more investigations to fully understand the molecular mechanisms, structure-function relationships and mechanisms of action of bacteriocins for exploration of applications should be performed.

Compliance with ethical standards

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Disclosure of conflict of interest

There is no conflict of interest.

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