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Research Article

Bacteriocins Production Potentials of some Gram Positive Cocci Bacteria Isolated from Milk and other Food Sources

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Abstract

Bacteria isolated from six different sources viz; cow milk, goat milk, sheep milk, burukutu, dawadawa and kunu- zaki were identified using Vitek 2 compact system. The identified isolates are *Staphylococcus lentus, Staphylococcus pseudintermedius, Staphylococcus sciuri, Kocuria kristinae, Kocuria rosea, Kocuria rhizophila, Enterococcus faecium, Enterococcus gallinarum, Enterococcus casseliflavus, Granulicatella elegans and Pediococcus pentosaceus.* Out of these isolates, *Enterococcus faecium, Enterococcus gallinarum, Enterococcus casseliflavus, Granulicatella elegans and Pediococcus pentosaceus* are lactic acid bacteria. The agar well diffusion method was used in testing the bacteriocins production potentials of all the isolates using *Staphylococcus aureus, Listeria monocytogenes, Salmonella typhi and Escherichia coli* as test organisms. Results showed that 20 out of the 23 isolates had antimicrobial activities. Isolate G8 (from goat milk) produced the highest zones of inhibition of 23mm against *Listeria monocytogenes* and 22mm against *Staphylococcus aureus.* B2 (isolate from burukutu) was the only isolate that inhibited all the test organisms. All the isolates produced acid though at varying degrees.

Keywords: Bacteriocin; vitek 2; gram positive cocci; lactic acid bacteria; kocuria

Introduction

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria that are capable of killing or inhibiting bacteria strains closely related or non-related to producing bacteria but will not harm the bacteria themselves due to their possession of specific immunity proteins [1]. Bacteriocins are capable of controlling clinically relevant susceptible and drug-resistance bacteria [2]. The vast majority of all bacteria species appear to be able to produce at least one bacteriocin, most of which are unknown. Bacteriocins producing bacteria could be extracted from various sources such as water, soil, animal intestines and food products. Bacteriocins may be secreted by both Gram positive and Gram negative bacteria. In particular, among Gram positive bacteria, bacteriocins derived from lactic acid bacteria are of great interest [3]. Reports on the first bacteriocin production were from *Escherichia coli* in 1925, and this peptide was named "colicins" to reflect the microbial source [4]. Bacteriocins are named according to the group of the producing bacteria, thus pediocins are produced by *Pediococci sp* [5], staphylococcins produced by *Staphylococci sp* [6], enterocins produced by *Enterococci* [7,8].

In recent years, a group of antibacterial proteins produced by Gram positive bacteria have attracted great interest in their potential use as food preservatives and as antibacterial agents to combat certain infections due to Gram positive pathogenic bacteria [9]. Bacteriocins of Gram positive bacteria are as abundant and even more

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diverse as those found in Gram negative bacteria. Firstly, bacteriocin production is not necessarily lethal to the producing cell due to the transport mechanisms Gram positive bacteria encode to release bacteriocin toxin. Typically, their biosynthesis is self-regulated with specifically dedicated transport mechanisms facilitating release, although some employ the sec-dependent export pathway. Secondary, the Gram positive bacteria have evolved bacteriocin-specific regulation, whereas bacteriocins of Gram negative bacteria rely solely on host regulatory networks [10]. Given that bacteriocins are produced by both Gram positive and Gram negative bacteria from diverse sources, this work therefore, examines Gram positive cocci isolated from different milk and food sources for their ability to produce bacteriocins against *Staphylococcus aureus, Listeria monocytogenes, Salmonella typhi and Escherichia coli*.

Methodology

Isolation and Identification

Fresh cow milk, goat milk, sheep milk, dawadawa (fermented cooking condiment made from locust beans seeds), burukutu (a locally prepared alcoholic beverage made from guinea corn grains) and kunu- zaki (a locally prepared non-alcoholic beverage made from guinea corn grains) were collected in sterile containers and taken to the laboratory and allowed to ferment for 24 hours. Serial dilutions were made for all the samples and appropriate dilutions (10⁻⁵ to 10⁻⁹) inoculated on MRS agar (Dextrose-20, Agar 12., Proteose peptone-10, Beef extract-10, Yeast extract-5, Sodium acetate-5, Ammonium citrate-2, Dipotassium phosphate-2, Tween 80-1, Magnesium sulphate-0.10 and Manganese sulphate-0.05 all g/L/, pH-6.5 at 250C) TITAN BIOTECH LTD India for 24 to 48 hours at room temperature (30-37°C). Discrete colonies observed were sub-cultured on MRS agar to obtain pure isolates. The pure isolates were Gram stained and the Gram-positive cocci bacteria isolates were examined further. The Gram positive cocci bacteria isolates were further identified using Vitek 2 compact system (BIOMERIEUX, France).

Four test organisms comprising two Gram positive bacteria (*Staphylococcus aureus and Listeria monocytogenes*) and two Gram negative bacteria (*E. coli and Salmonella typhylum*) were used to test for the bacteriocins production abilities of the isolates. For the preparation of crude bacteriocins, 10ml of MRS broth was inocu-

lated with 0.1ml (McFarland standard of 0.5) of freshly prepared cultures of each isolates and then incubated at 370^c for 24 hr. The 24 hr cultures were centrifuged at 6000rpm for 20min. The supernatants were filtered using cellulose acetate paper of pore size of 0.02µm to obtain cell free extracts [11,12]. The pH of the extract was adjusted to 5.5 with 1% sodium hydroxide solution. To determine the bacteriocin activity, agar well diffusion method was used. The four test organisms were then inoculated on Muller Hilton agar (Merck, Germany) and a sterile cork borer (6mm diameter) was used to bore holes on the agar surfaces. Consequently, 50µl of the extract was pipetted into the appropriate holes and allowed to absorb, then incubated for 24 hours at 37°C [13,14]. After 24 hr, zones of inhibition where present were measured and recorded in millimeter (mm) [15]. To test for the acid producing abilities, the isolates were grown in MRS broth with an initial pH of 6.2 and incubated at room temperature for 5 days. The pH of each isolate in the broth was measured with a pH metre (pHep HANNA instruments) at 24 hourly intervals, i.e. 24, 48, 72, 96 and 120 hr.

Results

Results of Gram-positive cocci isolated from cow milk, goat milk, sheep milk, dawadawa, burukutu and kunu-zaki is shown in the table below. KEY: B2 and B3 (Burukutu isolates); C2, C4, C9 and C10 (Cow milk isolates); D3, D5 and D8 (Dawadawa isolates); G1, G2, G4, G6, G7 and G8 (Goat milk isolates); K1, K3, K4, K5 and K6 (Kunu- zaki isolates and S1, S2 and S5 (Sheep milk isolates) Table 1 above contained 23 Gram positive cocci consisting of Staphylococcus pseudintermedius (4), Staphylococcus lentus (3), Staphylococcus sciuri (1), Kocuria kristinae (3), Kocuria rhizophila (1), Kocuria rosea (1), Enterococcus faecium (2), Enterococcus gallinarum (2), Enterococcus casseliflavus(1), Granulicatella elegans (1), Pediococcus pentosaceus (1) and unidentified isolates (3). Results of Antimicrobial activity of the crude bacteriocins from the isolates against Listeria monocytogenes, Staphylococcus aureus, E. coli and Salmonella sp are shown in the table below. Table 2 above showed the results of the crude bacteriocins against Listeria monocytogenes, Staphylococcus aureus, E. coli and Salmonella sp. Figures 1 - 5 below show the variations in pH of MRS broth inoculated with the isolates and monitored at 24 hourly intervals over a period of 5 days The results of the acid production profile of the isolates are shown in (Figures 1-5). All the isolates produced acid as measured at 5- day intervals.

 Table 1: Gram positive cocci bacteria from different sources identified with Vitek 2 compact system.

S/N	Code	Source	Identity	
1	B2	Burukutu	Staphylococcus lentus	
2	B4	"	Enterococcus casseliflavus	
3	C2	Cow milk	Kocuria rhizophila	
4	C4		Unidentified	
5	С9	, n	Staphylococcus sciuri	
6	C10	, n	Enterococcus gallinarum	
7	D3	Dawadawa	Pediococcus pentosaceus	



8	D5		Unidentified
9	D8	"	Unidentified
10	G1	Goat milk	Staphylococcus lentus
11	G2	"	Staphylococcus peudintermedius
12	G4	"	Kocuria kristinae
13	G6	"	Staphylococcus peudintermedius
14	G7	"	Enterococcus faecium
15	G8	"	Staphylococcus peudintermedius
16	К1	Kunu-zaki	Staphylococcus lentus
17	КЗ	"	Kocuria kristinae
18	К4	"	Enterococcus faecium
19	К5	"	Enterococcus gallinarum
20	К6	"	Granulicatella elegans
21	S1	Sheep milk	Kocuria rosea
22	S2	"	taphylococcus peudintermedius
23	S5	"	Kocuria kristinae

Fable 2: Zones of inhibition	produced by cel	l free extracts of the	e isolates against the	test organisms.
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S/N	CODE	SOURCE	ZONES OF INHIBITION ((in mm)			
			LIS	STA	ECO	SAL
1	B2	Burukutu	17	13	12	12
2	B4	"	14	12	-	-
3	C2	Cow milk	13	-	-	-
4	C4	,,	13	12	-	-
5	С9	,,	13	16	-	-
6	C10	,,	10	13	-	-
7	D3	Dawadawa	15	-	-	-
8	D5	"	12	13	-	-
9	D8	"	22	22	-	-
10	G1	Goat milk	15	12	-	-
11	G2	"	11	18	-	-
12	G4	,,	17	12	-	-
13	G6	,,	21	14	-	-
14	G7	,,	15	12	-	-
15	G8	,,	23	18	-	-
16	K1	Kunu-zaki	-	-	-	-
17	К3	,,	12	-	-	-
18	K4	"	14	12	-	-
19	K5	,,	-	-	-	-
20	K6	,,	-	-		-
21	S1	Sheep milk	10	11	-	-
22	S2	,,	20	17	-	-
23	S5	"	20	11	15	-

KEY: - = No inhibition, LIS= *Listeria monocytogenes*, STA= *Staphylococcus aureus*, ECO= *E. coli* and SAL= *Salmonella sp*









Figure 2: pH changes in the MRS broth containing *Staphylococcus lentus* (G1, B2, K1) and Staphylococcus sciuri (C9) isolates in MRS at 24- hour intervals over a-5-day period.



Kocuria rhizophila (C2) isolates at 24- hour intervals over a-5-day period.









Discussion

A total of eleven different Gram-positive cocci bacteria were identified using Vitek 2 compact system from the six different sources (Table1). The isolates includes Staphylococcus pseudintermedius, Staphylococcus lentus, *Staphylococcus sciuri, Kocuria kristinae, Kocuria rosea, Kocuria rhizophila, Enterococcus gallinarum, Enteerococcus faecium, Enterococcus casseliflavus, Pediococcus pentosaceus and Granulicatella elegans*. Gram positive cocci bacteria have been isolated from different sources. [16], isolated Gram positive cocci namely *Staphylococcus species, Streptococcus species* and *Lactococcus species* from burukutu. [17], also isolated some Gram positive cocci, *Streptococci* species and *Staphylococci species* from dawadawa produced from soya bean seeds. [18], isolated Gram positive cocci, *Staphylococcus species and Streptococcus species* from kunun-zaki. [19], also isolated Gram positive cocci namely *Staphylococcus, Streptococcus, Enterococcus, Micrococcus* and *Lactococcus species* from samples of raw milk collected from three areas of high milk production in Zulia state, Venezuela. Similary, [20], isolated *Lactococcus, Pediococcus, Leuconostoc, and Streptococcus species* from raw milk, cheese and yogurt.

Lactococcus, Enterococcus, Leuconostoc, Pediococcus and Streptococcus species were also identified from raw cow milk [21,22], isolated *Enterococcus mundtii*, a Gram-positive coccus bacterium from goat milk. Lactic acid bacteria were isolated from all



the samples except sheep milk (Table 1). Isolate B4 (*Enterococcus casseliflavus*), isolate C10 (*Enterococcus gallinarum*), isolate D3 (*Pediococcus pentosaceus*), isolate G7 (*Enteerococcus faecium*,) and isolate K4, K5 and K6 (*Enteerococcus faecium*, *Enterococcus gallinarum and Granulicatella elegans*) respectively [23], reported that lactic acid bacteria are ubiquitous bacteria group that are widely spread in nature in niches of diary (fermented), meat and vegetables origin, the gastrointestinal tract and urogenital tract of humans and animals, soil and water. [24], isolated *Enterococci species (Enterococcus faecium and E. faecalis.*) from a large variety of fresh and prepared foods of animal origin during routine microbiologic control tests in a distribution firm. Similary, [25], isolated *Pediococcus pentosaceous* from Satar Three isolates, C4, D5 and D8 were unidentified using Vitek 2 compact system (Table 1) apparantly because the organisms are not in the database of the machine.

The catalase test showed that C4 was catalase negative while D5 and D8 were catalase positive. However, microscopic examinations revealed them to be Gram positive cocci. Other methods of identification such as molecular could be explored to get the organisms fully identified. Except for K1, K5 and K6 (Table 2), all the isolates produced substances (bacteriocins) that inhibited the growth of some of the test organisms. This agrees with the report of that the vast majority of all bacterial species appear to be able to produce at least one bacteriocin, most of which are unknown. Generally, Gram positive test organisms were more susceptible to the inhibitory substances (bacteriocins) as each of the substances inhibited either Listeria monocytogenes or Staphylococcus aureus or both. Bacteriocins are known to be proteinaceous or peptidic toxins produced by bacteria to inhibit the growth of similar or closely related bacteria strain(s) [26]. Isolate G8 produced the highest zones of inhibition (23mm) against Listeria monocytogenes and (18mm) against Staphylococcus aureus followed by isolate D8 which produced (22mm) against Listeria monocytogenes and Staphylococcus aureus, followed by G6 with (21mm) against Listeria monocytogenes and (14mm) against Staphylococcus aureus.

Isolate S2 is forth with (20mm) against Listeria monocytogenes and (17mm) against Staphylococcus aureus, S5 with (20mm) against Listeria monocytogenes and (11mm) against Staphylococcus aureus while C9 with (12mm) against Listeria monocytogenes and (16mm) against Staphylococcus aureus and others. The variations in the zones of inhibition by the different cell free extracts on the test organisms could be a reflection of differences in the types and mode of actions of their bacteriocins content (Perez-Ramos et al., 2021) Isolate B2 produced inhibitory substances that has broad spectrum antimicrobial activities as it inhibited both the Gram-positive (Listeria monocytogenes and Staphylococcus aureus) and the Gram negative test organisms (E.coli and Salmonella sp). Isolate S5 also produced antimicrobial substance that has antimicrobial activity against E. coli in addition to Listeria monocytogenes and Staphylococcus aureus. Bacteriocins are known to have a narrow spectrum by inhibiting bacteria taxonomically close or a broad spectrum by inhibiting a wide variety of bacteria [27]. Among the isolates with

high antimicrobial activity are *Staphylococcus pseudintermedius* followed by D8 (partially unidentfied), *Kocuria kristinae* and *Staphylococcus scuri*. However, *Staphylococcus pseudintermedius* is the most frequently occurring isolate [28,29].

Reported that Staphylococcus psudintermedius strain 222 produces a novel peptide bacteriocin termed BacSp222 that inhibited methicillin-resistant S.aureus. Similaly, [30], reported the production of bacteriocin-like substance by Staphylococcus pseudintermedius S28 that inhibited isolates of S. aureus from foods showing inhibition zones of 14.2 to 28.3mm. The unidentified D8 produced antimicrobial substance that has high inhibitory activity next to Staphylococcus pseudintermedius. The lactic acid bacteria isolates: B4, C10, G7 and K4 which are Enterococcus species produced the antimicrobial substances that inhibited either Listeria monocytogenes or Staphylococcus aureus or both. Enterococcus species are known to be one of the most frequent producers of bacteriocins (enterocins), which confer them an advantage to compete in their natural environment (Almeida-Santos et al., 2012). Bacteriocins produced by Enterococci are often similar to those by other lactic acid bacteria (Ness et al., 2014) [31], reported that an isolate named E. faecium LCW 44 from raw camel milk exhibited a broad antibacterial spectrum with an inhibitory activity against several Gram-positive strains belonging to the genera Clostridium, Listeria, Staphylococcus, and Lactobacillus.

Also, Enterococcus casseliflavus M1001 has been shown to produce bacteriocins that have antimicrobial activity against Staphylococcus aureus [32]. Pediococcus pentosaceus (isolate D3) produced an antimicrobial substance that inhibited Listeria monocytogenes with zone of inhibition of 15mm. Pediococcus pentosaceus 147 isolated from artisanal Minas cheese produced with raw milk, and characterized as a bacteriocinogenic strain showed inhibitory activity against different strains of Listeria species (Gutierrez-Cortes et al., 2018). The Kocuria species (isolates C2, G4, K3, S1 and S5) produced antimicrobial substances that inhibited the growth of either one or both of the Gram positive test organisms. Kocuria varians NCC 1482 has been reported to produce a bacteriocin called variacin, which may be applied in food preservation without posing any safety or regulatory issue [33]. The unidentified isolates (D8, D5 and C4) by Vitek 2 compact system, however, produced antimicrobial substances that inhibited the growth of the two Gram positive test organisms. Isolate D8 produced high zones of inhibition (22mm) against both Listeria monocytogenes and Staphylococcus aureus. Isolate D5 has 12mm and 13mm against Listeria monocytogenes and Staphylococcus aureus while C4 produced zones of inhibition of 13mm and 12mm against Listeria monocytogenes and Staphylococcus aureus respectively.

The results of the acid production profile of the isolates are shown in Figures 1-5. All the isolates produced acid as measured in 5 days intervals. However, the acid production varied as shown by the pH readings. Figure 1 presents pH of isolates G2, G6, G8 and S2. These are all members of *Staphylococcus psudintermedius*. Figure



2 presents pH of isolates G1, B2, K1 and C4. Isolates G1, B2 and C4 are members of *Staphylococcus lentus* while G1 is *Staphylococcus sciuri*. [34]. reported that the genera *Staphylococci* that had an average GC content of 30-39%, produced acid from dextrose and the MRS agar used (TITAN BIOTECH LTD, India) contained 20g/ltr of dextrose thus this agreed with their report. The average GC content of *Staphylococcus psudintermedius* group genome ranges from 37.4 to 38.3% [35], while the genome sequence of *Staphylococcus lentus* F1142 has GC content of 31.8% [36]. Figure 3 presents the pH changes of the isolates G4, S5, K3, S1 and C2 which are Kocuria *species* (Kocuria kristinae, K. rosea and K. rhizophila).

Kocuria species are able to use various carbon sources to produce acids from one or more carbohydrates [36]. Figure 4 presents' pH changes of the isolates G7, K4, B4, C10 and K5 which are Enterococcus species (Enterococcus faecium, E. casseliflavus and E. gallinarum). Finally, Figure 5 presents changes in pH of isolates D3, K6, C4, D5 and D8, (Pediococcuss pentosaceus, Granulicatella elegans and the three unidentified isolates). Enterococcus species. Pediococcus pentosaceus and Granulicatella elegans are all members of lactic acid bacteria. Lactic acid bacteria are a group of Gram positive, non- spore forming, facultative anaerobes, catalase negative cocci or rods which produce lactic acid as the major end product of fermentation of carbohydrates [37-39]. The changes from initial acidity to alkalinity with the bacterial growth in MRS medium (Figures 1,3 & 4) that correlate with the formation of white precipitates, which increased with increase in alkalinity, is likely due to reactions among the intermediate products of LAB fermentation or the products with the medium component. The nature of precipitate is presently unknown.

Conclusion

Bacteriocin genic Gram positive cocci bacteria are associated with burukutu, cow milk, dawadawa, goat milk, kunu-zaki and sheep milk. The Gram positive cocci bacteria isolated from these samples include genera of *Staphylococcus, Kocuria. Enterococcus, Granulicatella and Pediococcus.* Their cell free extracts ability to inhibit growth of test organisms with either narrow or broad spectrum activity indicates their bacteriocin producing potential. There is a need to identify, purify and classify the bacteriocins produced by these group of organisms to ascertain their safety and range of activity. The organic acid (s) produced by non-lactic acid bacteria needs to be identified as this may assist in the classification of these genera of bacteria. This research also shows that all the isolates produced acid from dextrose. However, there is apparently no correlation between bacteriocins and acid production.

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