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**RESEARCH ARTICLE**

# Antimicrobial Efficiency of Purified and Characterized Bacteriocins Produced by *Lactobacillus bulgaricus* Y34 and *Lactococcus lactis* N22 Isolated from Fermented Milk Products

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## *Abstract*

*Lactobacillus bulgaricus* Y34 and *Lactococcus lactis* N22 were propagated in De Man Rogosa Sharpe (MRS) broth separately for bacteriocin production. There were variations in bacteriocin activity, total activity, specific activity and protein content during the process of bacteriocin purification. After the stages of purification of bacteriocins produced, *L. lactis* N22 had higher bacteriocin recovery (9.5%) while *L.bulgaricus* Y34 had 8.8% bacteriocin recovery. Bacteriocins produced by *L. bulgaricus* Y34 and *L. lactis* N22 exhibited bacteriocin activity of 6000±0.00 and 5800±0.00 AU/mL respectively.The bacteriocins were stable, slightly stable and unstable at various exposed, storage temperatures and at different pH levels. The bacteriocins produced by *L. bulgaricus* Y34 and *L. lactis* N22 were identified as bulgarican and nisin respectively. The bulgarican and nisin produced by the *L.bulgaricus*Y34 and *L. lactis* N22 inhibited these spoilage pathogenic microorganisms: *Staphylococcus* sp N23, *Salmonella* sp N17, *Bacillus* sp W12, *Shigella* sp N25 and *Pseudomonas* sp W1 under study.Bacteriocins play very important role in food preservation processes by inhibiting or killing of spoilage and/or pathogenic microorganisms in food and dairy products.



## Introduction

Lactic acid bacteria (LAB) are a group of gram-positive bacteria, non-spore forming, non-respiring, cocci or rods, which produce lactic acid as the major end product of the fermentation of carbohydrate. Historically, bacteria from the genera *Lactobacillus,Lactococcus, Leuconostoc, Pediococcus* and *Streptococcus* are the main species involved. Several more have been identified but minor role in lactic fermentations (Aexlson, 1998).LAB provide protection against spoilage microorganisms by producing varieties of antimicrobial compounds, including bacteriocins and also due to pH decrease and competition for substrates. LAB produce various compounds such as organic acid and bacteriocin during lactic fermentation (Lindgren and Dobrogosz, 1990).

Bacteriocin are naturally occurring antibiotic peptides produced by Gram positive bacteria and may contain as much as 24 amino acids. Some Bacteriocins are lantibiotics, which means that they are post translationally modified so as to encompass the amino acid lanthionine or "Lan" (Chattejee *et al.,* 2005). In general, bacteriocins are cationic peptide that display hydrophobic or amphiphilic properties and the bacterial membrane is in most cases the target for their activity. Bacteriocins have recently been grouped in to three classes (class I: lantibiotics such as nisin produced by *Lactococcus lactis,* class II: Small heat non-lantibiotics such as pediocin produced by *Pediococcus* sp class III: Small heat labile proteins such as helveticin J produced by *Lactobacilus helveticus*) on the basis of the sequence

of the nature peptides and prepeptides (Mortvedt *et al*., 1996). Some bacteriocins kill only bacterial belonging to the same species as producers, one word other bacteriocins kill a broad range of gram positive bacteria (Coventry *et al*., 1997., Ennahar *et al*., 2000). The incorporation of these compounds as bio preservative agent into model foods has been shown to be effective in the control of pathogenic and spoilage microorganisms (O"Sullivan., *et al.,* 2002). Devugst and Vandamme (1994) reported that nisin produced by *Lactococcus lactis subsp lactis* was the first bacteriocin used on commercial scale as food preservative and dates back to the first half of this century. Bacteriocins produced by *Lactobacillus acidophilus* can be used for the treatment of diarrhea in children. They may aid in the management of chronic or persistent diarrhea and bacteria over growth related diarrhoea (Farkas-Himskey and Yu, 1985). Other bacteriocins of *Lactobacillus* has been reported to be effective against closely related species of mesophilic *Lactobacillus* and therefore considered as potential natural food preservatives (Daeschel, 1993., De Vugst and Vandamme, 1994).

The aim of this study is to assess the antimicrobial efficiency/potentials between the purified and characterized bacteriocins produced by the lactic acid bacteria of the species of *Lactobacillus bulgaricus* Y34 and *Lactococcus lactis* N22 on some selected microbial isolates.

## MATERIALS AND METHODS

### Collection of Samples

Five (5) Samples each of yoghurt and nono (Fermented milk) were collected from Bosso – Nigeria in sterile sample bottles and were transferred to the laboratory in ice box for isolation of lactic acid bacteria and indicator microorganisms.

### Isolation ,Characterization and Identification of Lactic acid bacteria and indicator microorganisms.

Serially diluted samples of the fermented milk products were inoculated on to lactic acid agar medium, LAM (agar-agar 15g, tryptone 20g, yeast extract 5g, gelatin 2.5g, glucose 5g, lactose 5g, and sucrose 0.52g) and nutrient agar for isolation of indicator microorganisms and incubated at $37^0$C for 24-48 hours. Colonies which appeared on the plates were counted using colony counter (Model 6399 manufactured by Stuart Scientific Co. Ltd., Great Britain) and the results were recorded as colony forming units per milliliter (cfu/ml) of sample. Pure isolates were characterized based on colony morphology, cell morphology and biochemical tests (Fawole and Oso., 1998, Oyeleke and Manga, 2008). The isolates were identified using the scheme of Cheesebrough (2003).

### Production of bacteriocins

*Lactobacillus bulgaricus* Y34 and *Lactococcus lactis* N22 were propagated in 1000ml of De Man Rogosa Sharpe (MRS) broth at pH 5.8, temperature of $30^o$C for 48 hours. For extraction of bacteriocins, a cell free solution of bacteriocins was obtained by centrifugation at 10,000 rpm for 20 minutes. The cultures were then adjusted to pH 7.0 using NaOH to exclude the antimicrobial effects of organic acids, and then followed by filtration of the supernatant through 0.2µm pore size cellulose acetate filter (Schillinger and Lucke, 1989).

### Purification of bacteriocins

The bacteriocins were purified by the method of Bradford (1976) and Jimenze-Diaz *et al.*(1993) using ammonium sulphate precipitation, trichloroacetic acid precipitation and ultra filtration analysis.

### Ammonium sulphate precipitation

The crude bacteriocins produced by *Lactobacillus bulgaricus* Y34, and *Lactococcus lactis* N22 were treated with solid ammonium sulphate (Mallinckrodth Clinical, Inc. Paris, France) to 30% saturation for each bacteriocin samples respectively. The mixture was stored at $4^o$C for 2 hours and centrifuged at 20,000 rpm for 1 hour. The precipitate was re-suspended in 25ml of 0.5ml potassium phosphate buffer (pH7.0). The suspension was dialyzed in tabular cellulose membrane against 2 liters of the buffer for 18 hours in dialysis tubing (Jimenez-Diaz *et al.,* 1993). The products of ammonium phosphate precipitation constituted fraction I.

### Trichloroacetic acid (TCA) precipitation

5% of TCA was added to 25ml of fraction I to precipitate the protein (bacteriocins). The mixture was centrifuged at 13,000 rpm for 10 minutes after which the supernatant was decanted. The resulting pellet was dissolved in 2ml of potassium phosphate buffer to obtain fraction II.

### Ultra filtration analysis

The fraction II bateriocin samples was re-suspended to 1/3 volume in potassium phosphate buffer (50m, M, pH 7.0). Several aliquots were ultra filtered through various filtron membranes (filtron Technology Corp; North –borough, Massachusetts), including 1,000, 10,000 KDa molecular sizes. Bacteriocin activity was determined (Jimenez-Diaz *et al.,* 1993) and protein concentrations of the fraction

were determined by the Bradford method (Bradford, 1976).

**Characterization of bacteriocins**
The purified bacteriocins produced by *Lactobacillus bulgaricus* Y34 and *Lactococcus lactis* N22 were characterized with respect to thermal stability, pH stability, stability during storage and other properties exhibited by bacteriocins produced (Rammelsberg and Radlar, 1990., Brinkten *et al*., 1994 and Soomro *et al.,* 2002).

**Temperature stability of bacteriocins**
400µl of purified bacteriocin samples were dispensed into different test tubes for each bacteriocin under test using pipette and were exposed to the following oven temperature: 40, 60, 80,100 and 121$^{o}$C. Aliquots of bacteriocins produced by *Lactobacillus bulgaricus* Y34 and *Lactococcus lactis* N22 exposed were removed after 30 minutes and were tested for bacteriocin activity against indicator microorganisms (*Staphylococcus* sp N23, *Salmonella* sp N17, *Shigella* sp N25, *Bacillus* sp W12 and *Pseudomonas* sp W1.

**pH stability of bacteriocins**
400 µl of purified bacteriocins were dispensed into different test tubes using pipette and bacteriocins were adjusted to pH 2, 4, 6,8,10 and 12 with hydrochioric acid (HCL) sodium hydroxide (NaOH) and were incubated at room temperature for 24 hours (Brinkten *et al.,* 1994). The bacteriocins were assayed against the indicator microorganisms.

**Stability of bacteriocins during storage**
Purified bacteriocins were kept in test tubes and stored at 4$^{o}$C and 10$^{o}$C (refrigeration temperature) and 30$^{o}$C (room temperature). Samples were withdrawn from the stored material after 24 hours to determine bacteriocin activity (Rammelsbarg and Radlar, 1990).

**Other properties of bacteriocins exhibited**
The purified bacteriocins properties were based an spectrum (broad, narrow), bactericidal, bacteriostatic characteristics (early or late in the cycle) and class of bacteriocins produced by *Lactobacillus bulganicus* Y34 and *Lactococcus lactis* N22 (Soomro *et al.,* 2002).

**Determination of bacteriocin activity**
Well assay procedures of Schillinger and Lucke (1989) and Takuhiro *et al;* (1991) were used. Aliquots of 500 µl from each bacteriocin produced by *Lactobacillus bulgaricus* Y34 and *Lactococcus lactis* N22 was placed separately in agar wells in Petri dishes seeded with the bioassay strain (indicator microorganisms *Staphylococcus* sp N23, *Salmonella* sp N17, *Shigella* sp N25, *Bacillus* sp W12 *and Pseudomona* sp W1) and incubated overnight at 37$^{o}$C. The diameter of the zone of inhibition (mm) was measured (Rammelsberg and Radlar, 1990). The antimicrobial activity of bacteriocins produced by test isolates was defined as the reciprocal of the highest dilution showing inhibition of microorganisms multipied by 100 and it is expressed as activity unit per milliliter (Au/ml) (Graciela *et al*., 1995).

**RESULTS**
*Lactobacillus bulgaricus* Y34 and *Lactococcus lactis* N22 were observed to be good bacteriocin producers after propagating the organisms in De man Rogosa Sharpe broth, producing growth ability of 0.90 each, pH 3.91 and 3.70, and bacteriocin activity (AU/mL) of 6000$\pm$0.00 and 5800$\pm$0.00 respectively (Table 1). *Lactobacillus bulgaricus* Y34 had higher bacteriocin activity (6000$\pm$0.00 AU/mL) than *Lactococcus lactis* N22 (5800$\pm$0.00 Au/ml). It was observed that there were variations in bacteriocin activity, total activity, specific activity and protein content of bacteriocin produced by the organisms. It was also observed that the specific activity, amount of protein and recovery of bacteriocins was decreasing after each treatment of bacteriocins (Table2). *Lactococcus lactis* N22 had the highest bacteriocin recovery (9.5%) while *Lactobacillus bulgaricus* Y34 had 8.8% bacteriocin recovery at the end of the purification process (Table 2).Bacteriocin produced by *Lactococcus lactis* N22 was most heat stable at 40$^{o}$C, 60$^{o}$C and 80$^{o}$C for 30 minutes, but slightly stable at 100$^{o}$C and unstable at 121$^{o}$C while that of *Lactobacillus bulgaricus* Y34 was heat stable at 40$^{o}$C and 60$^{o}$C, but slightly stable at 80$^{o}$C and unstable at 121$^{o}$C for 30 minutes (Table3). Bacteriocins produced by *L. bulgaricus*Y34 maintained full stability after storage for 60 days at 4$^{o}$C, slightly stable at 10$^{o}$C and unstable at 30$^{o}$C while bacteriocins produced by *L. lactis* N22 was stable at 4$^{o}$C, slightly stable at 10$^{o}$C and unstable at 30$^{o}$C storage temperature after 60 days (Table3). It was observed that bacteriocins produced by *L. bulgaricus* Y34 and *L. lactis* N22 were stable at pH 2,4 and 6, slightly stable at pH 8 and unstable at pH 10 and 12 (Table3).
The bacteriocins obtained also exhibited some properties that justified their characterization; these properties include broad spectrum of inhibition of indicator microorganisms by both *Lactobacillus bulgaricus* Y34 and *Lactococcus lactis* N22. Bacteriocin was produced early in the growth cycle by *L bulgaricus* Y34 and late in the growth cycle by *L. lactis* N22. Bacteriocin from both organisms were bactericidal (Table3). Based on the characteristics of

the bacteriocins produced by the test isolates, they were suspected to be bulgarican (produced by *L. bulgaricus* Y34) and nisin (produced by *L. lactis* N22) (Table3). The results revealed that bacteriocins produced by both *L. bulgaricus* Y34 and *L. lactis* N22 inhibited all indicator microorganisms (*Staphylococcus* sp N23, *Salmonella* sp N17, *Bacillus* sp W12, *Shigella* sp N25, and *Pseudomonas* sp W1) (Table4).The inhibitory effects observed by bacteriocin produced by the LAB against the indictor organisms might be due to stability of the bacteriocins at exposed and storage temperature, pH, bactericidal and bacteriostatic properties broad, spectrum and time of its production in the growth cycle and most importantly the bacteriocin activity at activity units per milliliter (AU/mL).

**Table 1. Production of bacteriocin by *Lactobacillus bulgaricus* Y34 and *Lactococcus lactis* N22**

| Coded Organisms | Growth of LAB (580nm) | pH of bacteriocins | Bacteriocin activity (Au/ml) |
|---|---|---|---|
| *Lactobacillus Bulgaricus* Y34 | 0.90 | 3.91 | 6000±0.00 |
| *Lactococcus lactis* N22 | 0.90 | 3.70 | 5800±0.00 |

Y: Isolate from yoghurt, N: isolate from nono, Au/mL: Activity unit per milliliter, nm: nanometer

**Table 2. Purification of bacteriocins produced by *Lactobacillus bulgaricus* Y34 and *Lactococcus lactis* N22**

| Coded Organisms | Purification stage | Volume of culture supernatant (ml) | Activity of bacteriocins (Au/ml) | [I]Total a activity | [II]protein (µg/ml) | [III]specific activity | [IV] Recovery (%) |
|---|---|---|---|---|---|---|---|
| *Lactobacillus Bulgaricus* Y34 | culture supernatant | 1000 | 5800 | 5800000 | 400 | 14.5 | 100 |
| | Ammonium sulphate Precipitation | 30 | 5800 | 174000 | 390 | 14.9 | 97.5 |
| | Trichloroacetic acid Precipitation | 5 | 5800 | 29000 | 70 | 82.9 | 17.5 |
| | Ultra filtration | 8 | 6000 | 48000 | 35 | 1000 | 8.8 |
| *Lactococcus Latis* N22 | Culture supernatant | 1000 | 5600 | 5600000 | 337 | 16.6 | 100 |
| | Ammonium sulphate Precipitation | 30 | 5600 | 168000 | 308 | 18.2 | 91.4 |
| | Trichloroacetic acid Precipitation | 5 | 5600 | 28000 | 45 | 124.4 | 13.4 |
| | Ultra filtration | 8 | 5800 | 46400 | 32 | 1933.3 | 9.5 |

[I]Total activity was determined by the multiplication of volume by activity
[II]Protein concentration was determined by the Bradford method
[III]Specific activity is the activity units divided by the protein concentration (µg/ml)
[IV]Recovery percentage is the remaining protein concentration as a percentage of the initial protein concentration.
Y: Isolate from yoghurt, N: isolate from nono

**Table 3. Characterization of bacteriocins produced by *Lactobacillus bulgaricus* Y34 and *Latococcus lactis* N22**

| Coded Organisms | Stability | Stability of bacteriocins at: Exposed Temperature($^oC$) | Storage Temperature($^oC$) | pH | Properties of bateriocins | Bacteriocins |
|---|---|---|---|---|---|---|
| *Lactobacillus bulgaricus* Y34 | Stable | 40,60 | 4 | 2,4,6,8 | Broad spectrum, bactericidal, | Bulgarican |
| | Slightly stable | 80,100 | 10 | 10,12 | Produced early in the growth cycle | |
| | Unstable | 121 | 30 | 10,12 | | |
| *Lactococcus Lactis* N22 | Stable | 40,60,80 | 4 | 2,4,6 | Broad spectrum, bactericidal, | Nisin |
| | Slightly Stable | 100 | 10 | 8 | Produced late in the growth cycle | |
| | Unstable | 121 | 30 | 10,12 | | |

Y: Isolate from yoghurt, N: isolate from nono

**Table 4. Inhibition of indicator microorganisms by bacteriocins produced by *Lactobacillus bulgaricus* Y34 and *Lactococcus lactis* N22**

| Indicator microorganisms | Source | Zone of inhibition (mm) caused by bacteriocins produced by *Lactobacillus bulgaricus* Y34 | *Lactococcus lactis* $N_{22}$ |
|---|---|---|---|
| *Staphylococcus* sp N23 | Nono | +(6mm) | +(6mm) |
| *Salmonella* sp N17 | Nono | +(6mm) | +( 6mm) |
| *Bacillus* sp W12 | Wara | +(6mm) | +(6mm) |
| *Shigella* sp N25 | Nono | +(6mm) | +(6mm) |
| *Pseudomonas* sp W1 | Wara | +(6mm) | +(6mm) |

+: inhibition,Y:yoghurt;N:nono;W:wara.

**DISCUSSION**

Bacteriocin activity of 6000±0.00 was exhibited by *Lactobacillus bulgaricus* Y34 and *Lactococcus lactis* N22 exhibited bateriocin activity of 5800±0.00 against *Staphylococcus* sp N23, *Salmonella* sp N17, *Bacillus* sp W12, *Shigella* sp N25 and *Pseudomonas* sp W1. This is similar to the findings of Ogunbanwo *et al* .(2003) that *Lactobacillus plantarum* F1 and *Lactobacillus brevis* OG1 exhibited bacteriocin activity between 3200 (Au/ml) and 6400±0.00 (Au/ml) against *Escherichia coli, Listeria monocytogenes and Enterococcus faecalis*. This is similar to Mohammed (2009) that Of all the LAB screened for bacteriocin production, *Lactobacillus bulgaricus* Y34, *L. lactis* W15, *L. acidophilus* N20, *Lactococcus lactis* N22, *Streptococcus thermophilus* Y27, *S.cremoris* W1, *Pediococcus halophilus* W9 and *P. cerevisae* N21 produced bacteriocin activity between 4800 and 6000AU/mL against *Staphylococcus sp*, *Salmonella sp*, *Bacillus sp*, *Shigella* sp *and Pseudomonas sp*.This findings also agrees with Mohammed (2012) that Of the total LAB screened also for bacteriocin production, *Lactococcus lactis* B2, *Lactobacillus bulgaricus* B5, *Lactobacillus acidophilus* F16, *Lactobacillus helveticus* B9, *Pediococcus halophilus* P20 and *L. mesenteroides* F12 produced bacteriocin activity between 5000 AU/mL and 6000 AU/mL against the indicator microorganisms (*Pseudomonas aeruginosa* F13a, *Escherichia coli* B3, *Staphylococcus aureus* P23, *Salmonella typhimurium* B8, *Bacillus cereus* F14 and *Klebsiella pneumoniae* P21). This is in conformity with Mohammed *et al.* (2012) who reported that pediocin and nisin produced by *Pediococcus halophilus* W9 and *Lactococcus lactis* N24 exhibited bacteriocin activity between 5000±0.00 and 5600±0.00 AU/mL against

*Pseudomonas aeruginosa,Shigella dysenteriae* and *Eschericia coli.*

The optimal bacteriocin recovery was achieved using ammonium sulphate precipitation and trichloracetic acid precipitation. This is in conformity with the report of Invanova *et al* .(2000) that optimal bacteriocin recovery can be achieved by ammonium phosphate precipitation and trichloroacetic acid precipitation. Ultra filtration experiments in this study showed that bacteriocin were unable to pass through 1000 KDa molecular weight cut off membrane. A tendency to aggregate with other proteins has been reported in bacteriocins produced by other lactic acid bacteria (Bhunia *et al.,* 1988., Toba *et al.,* (1991) Klaenhammer, 1998., and might have contributed to why the bacteriocins could not pass through the membrances with low molecular weight cut off.

The bacteriocin produced by *L. bulgaricus* Y34 and *L. lactis* N22 were heat stable at 20, 40, 60 and 80$^{o}$C in most cases but slightly stable at 100$^{o}$C and unstable at 121$^{o}$C for 30 minutes of exposure to heat. This is consistent with Anderson (1986) who reported loss of bacteriocin activity after heat treatment of bateriocin at 121$^{o}$C for 15 minutes.This agrees with Mohammed *et al*.(2013) that Bacteriocin produced by *Lactobacillus acidophilus* FCF2 was most heat stable when exposed at 40$^{o}$C, 60$^{o}$C and 80$^{o}$C for 30 minutes, slightly stable at 100$^{o}$C but unstable at 121$^{o}$C,these could be as a result of the stability of the Bacteriocin to the levels of heats it was exposed to and this could serve as basis for it selection for use in biopreservation studies,because for a bacteriocin to be use for biopreservation,it must have high stability to heat.Temperature stability is important if the bacteriocin is to be used as food preservative, because many procedures of food preparation involve heating steps. The activity of bacteriocin elaborated by the test isolates was also observed to be pH dependent at pH 8-12.

Reddy *et al.,* (1984) and Abdef-Bar *et al*.(1987) reported similar observation, that bacteriocins produced by *L. bulgaricus* were shown to have high activity and stability at pH 2, 4 and 6 respectively against a range of pathogenic and spoilage bacteria. Bacteriocins produced by the test organisms have some interesting characteristics.This agrees with Mohammed *et al*.(2013) that The activity of Acidophilin elaborated was observed to be pH dependent. The highest antibacterial activity was exhibited within pH range of 2 - 10, while inactivation occurred mostly at pH 12. The Bacteriocin also exhibited some properties that justified it characterization. These properties include broad and narrow spectrum inhibition by pathogenic and spoilage microorganisms, bactericidal effects and the time of Bacteriocin production by *Lactobacillus acidophilus* FCF2.

The most striking is that these bateriocins were limited by the extremely broad antimicrobial spectrum. This is similar to the findings of Halo *et al.,* (1991) and Barefoot and klean hammer, (1993) that bateriocins were limited by the extremely narrow antimicrobial spectrum for bacteriocin, of some lactic acid bacteria (LAB), for example, lactococcin A and Lactacin B. Both *L. bulgaricus* Y34 and *L. lactis* N22 exhibited large spectrum against the indicator microorganisms used in this study.

This agrees with earlier reports by Tagg *et al.* (1976), Daesched and kleanhammer (1985), and Sanni *et al.* (1999) that some bacteriocins produced by gram-positive bacteria have broad spectrum activities. How ever, it was generally observed that bacteriocin from the producer organisms had no inhibilitory effects on the organisms producing it.

The suspected bacteriocins were identified as bulgarican and Nisin produced by *Lactobacillus bulgaricus* Y34 and *Lactococcus lactis* N22 respectively. Brinkten *et al.,* (1994) and Soomro *et al.* (2002) had identified and characterized bacteriocins produced by *Lactococcus lactis subsp lactis* AT-CC11454, *Pediococcus pentasaceas* FCC61, *Pediococcus acidilactici* H, *Leuconostoc gelidyn* UAL187, *Lactobacillus helveticus* 481, and *Camobacterium piscicola* LV17 as nisin, pediocin A, pediocin ACH, lecucocin, helveticin J and camobacteriocin respectively.

In conclusion, bulgarican produced by *L. bulgaricus* Y34 was more effective on the indicator microorganisms used in this study than nisin produced by *L. lactis* N22, though nisin is widely used in food industries in most countries for bio preservation of food products. We therefore recommend both bulgarican and nisin to food processing industries for biopreservation of food products. These bacteriocins produced could serve as alternative to chemical preservatives/additives used in food preservation.

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