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In-Vivo Assessment of Anti mycobacterial Activity of Bacteriocins From Lactic Acid Bacteria In Milk

Nwanekwu Kenneth Emeka [1]*, Rose Nkechinyere Nwabueze[2], Justina Chinyere Orji[2]

1.Department of Microbiology, Imo State University, Owerri, Nigeria

2. Department of Microbiology, School of Biological Sciences, Federal University of Technology, Owerri.

ABSTRACT

The inhibitory activity of bacteriocins produced by Lactic acid bacteria isolated from raw cattle and goat milk and other processed milk products against *Mycobacterium bovis and Mycobacterium tuberculosis* in milk was studied using spectrophotometry. Six bacteriocin positive strains of LAB were isolated and identified as *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus fermentum, Lactobacillus brevis, Lactococcus lactis and Streptococcus thermophilus*. The antimycobacterial activity of the bacteriocins in milk showed that the bacteriocins from Lactobacillus inhibited the growth of the mycobacterium over a period of 15 days while bacteriocin from Streptococcus did not inhibit the *Mycobacteria* in milk. However, the activity of the bacteriocin were lost after the 15th day as shown by increase in absorbance on day 18. The bacteriocin mix from all six bacteriocins against *Mycobacteria* was the most efficient when compared to that of individual bacteriocins, recording a significant decline in absorbance at from 0.79 to 0.026 even on day 18.

Keywords: Absorbance, bacteriocin, Lactic acid bacteria, milk, mycobacterium, spectrophotometry

*Corresponding Author Email: kennwanekwu@yahoo.com

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INTRODUCTION

Bacteriocins are proteinaceous substances mainly produced by Lactic acid Bacteria (LAB) with antibacterial activity against organisms of related species or across different genera. There is a renewed interest in bacteriocin due to its potential application in food industry and as biopreservatives especially in milk and dairy products. Milk provides a good ecosystem for microbial growth based on the amount of nutrient contained and so has the potential to harbour lots of both beneficial and pathogenic organisms including Mycobacterium. These contaminating microorganisms may be derived from a site of infection within the milk-producing animal itself. Consumption of such contaminated milk can give rise to diseases such as Brucellosis, Q fever, Listeriosis and Tuberculosis. [1-4]

Tuberculosis is typically a disease of the lungs caused by *Mycobacterium tuberculosis* complex. *Mycobacterium* comprises organisms that are gram–positive rods, aerobic to microaerophilic, non-motile, asporogenous bacteria that are acid fast such as *M. tuberculosis, M. bovis, M. africanum, M. microtii and M. canetii.* Tuberculosis is the second most common cause of death from infectious disease and roughly one-third of the world's population has been infected with *M. Tuberculosis.* Most cases worldwide are caused by *M. tuberculosis*. However, *M. africanum and M. canettii,* are seen less often and are more restricted to patients from sub-Saharan Africa. Variants most closely associated with animal hosts but known to also infect humans are *M. bovis* from cattle and *M. caprae* from goats. Presently, the disease has assumed an epidemic proportion in some parts of the world, and about 32% of the world's population is already infected with the causative agents. Prevalence of pulmonary TB due to *M.bovis* in certain parts of Africa has been reported with increasing frequency. A more worrisome impact of the disease is felt in its zoonotic status as it affects a range of food animals including cattle. This thus broadens the damage the disease is capable of, given the fact that the infectivity ratio in humans is significantly increased through the animal infection. *Mycobacterium bovis* is responsible for over 90% of animal cases of tuberculosis, while *M. tuberculosis* is responsible for 10%. In Nigeria, the prevalence of pulmonary tuberculosis from *M. bovis* varies from 10% in the southern part of the country to about 15% in the more nomadic north. Survey from the northern region of Nigeria compared isolates of *M. bovis* isolated from humans and cattle and found the isolates from both to be genetically identical. *M. bovis* and *M. tuberculosis* have been isolated from the milk of tuberculosis positive cattle in Nigeria, and also from unpasteurised milk commercially available. A herd prevalence of 10% has been observed within Nigeria and this indicated the presence of bovine tuberculosis in the herds in the northern region, thus confirming the endemic nature of the disease in Nigerian cattle. This indicates serious Public Health implications.

More so, milk and meat have been incriminated as one of the most important links between bovine tuberculosis and human beings particularly in developing countries like Nigeria where the disease is still a major problem with no control measures observed. There is therefore need to tackle this problem by inhibiting the proliferation of the organism in milk and its product with the use of safe and effective products. [5-17]

Today, consumers are concerned about the synthetic chemicals used as preservatives in food, and there is resulting trend towards less processed food. A solution to this dilemma is the use of antimicrobial metabolites of fermentative microorganisms because of their safe association with human fermented foods. It is therefore in line to state that the aim of this work is to assess the In vivo inhibitory effect of bacteriocin from LAB against *Mycobacterium sp* introduced into milk. [18-19]

MATERIALS AND METHOD

Isolation of Lactic Acid Bacteria (LAB) from Milk

LAB were isolated from different milk samples according to the method described by Kos et al. One hundred (100) ml of the liquid milk samples were collected in sterile conical flasks and allowed to ferment at room temperature for 3 days. Ten fold serial dilutions of the samples were made and 0.1ml of suitable dilution inoculated unto MRS agar. The pH of the medium was adjusted to 5.5 by adding HCl. The set plates were incubated anaerobically at 35^{o}C for 24-48hours. Colonies were tested for catalase activity. Catalase negative organisms were subcultured on fresh sterile MRS Agar to obtain pure culture. The isolated microorganisms were sub-cultured unto a maintenance culture medium of MRS broth containing 12% v/v glycerol. This was incubated at 30^{o}C until growth was detected and then stored at 4^{o}C in refrigerators. [20]

Identification of Isolates

The isolates were identified using standard microbiological methods. This includes Morphological, Physiological and Biochemical Tests. [21]

Preparation of Crude Bacteriocin

Bacteriocin was prepared by the method described by Kos et al. Broth cultures of all the LAB isolates were first prepared by simply inoculating a loopful of culture into fresh 20ml MRS broth in 25ml sterile bottles and incubated at 35^{o}C in an anaerobic jar for 48-72 hours. The 72 hour broth culture of LAB was used in the preparation of crude bacteriocin. About 10ml of the LAB broth culture was transferred into tubes and centrifuged at 5000 revolution per minute (r.p.m) for 15 minutes to obtain clear sedimentation of the pellets. The supernatant was decanted into separate containers. The supernatant fluid was adjusted to pH 6.5 by adding NaOH and then treated with 5mg/ml catalase.

The supernatant fluid was then filter sterilized through a 0.45µm pore size cellulose acetate filter. The product was designated as crude bacteriocin. [20]

Antimycobacterial Activity of Bacteriocin against *Mycobacterium* sp. in Milk

The antimycobacterial assay was carried out according to the method of Cadmus and Adesokan. Twenty micro litre of bacteriocin fluid was added to 20ml of pasteurised milk in a screw-capped bottle. Then, 0.1ml of *M. Bovis* culture was inoculated into the milk in the bottle. To another bottle containing 20ml of milk, 20ul of a mixture of all six bacteriocin fluids was added and inoculated with 0.1ml of *M. bovis* culture. The set up was incubated at 35^{o}C for 18days. Growth of *M. bovis* was monitored at 72 hours interval for 18 days. This was done spectrophotometrically by measuring the absorbance (optical density) using a spectrophotometer. Also, the antimycobacterial activity of a mixture of the 6 bacteriocins were done and compared with that of the individual bacteriocins. [5]

RESULTS AND DISCUSSION

Isolation and Identification of Bacteriocin Positive LAB Isolates

Table 1 shows the characteristics of the bacteriocin positive LAB isolates labelled LB I-VI with respect to growth temperature, catalase test, nitrate reduction, Gram reaction, spore formation, microscopy, growth in Sodium Chloride (NaCl) and motility and the fermentation pattern of the six isolates on different sugars. All the six LAB isolates were catalase negative, nitrate reductase negative, non motile, and did not form spores. Isolate I was Gram positive rod and showed growth in 5% NaCl, and at temperature 45^{o}C but no growth at 10^{o}C. Isolate II was observed as Gram positive rods which grew at 45^{o}C. No growth was observed at 10^{o}C and in 5% NaCl. Isolate III was identified as Gram positive cocci (in chains) and had no growth at 10^{o}C and45^{o}C but grew in 5% NaCl. Isolate IV was identified as Gram positive cocci which didn't grow at 45^{o}C and 10^{o}C but Isolate V was identified as Gram positive coccobacilli which grew in 5% NaCl and at 45^{o}C but not at 10^{o}C. Isolate VI was identified as Gram positive coccobacilli which grew in 5% NaCl and at 45^{o}C and 10^{o}C. The isolates displayed varied fermentation abilities of the sugars used. From the results, LAB I was identified as *Lactobacillus acidophilus*, LAB II as *Lactobacillus casei*, LAB III as *Streptococcus thermophilus*, LAB IV as *Lactococcus lactis*, LAB V as *Lactobacillus fermentum* and LAB VI as *Lactobacillus brevis*.

Table 1: Morphological and Biochemical Characteristics of Bacteriocin Positive LAB Isolates

Isolate	Gram stain	shape	Spore stain	Motility	cat	Nit	Growth at 10ºC	Growth at 45ºC	Growth at 5% NaCl	lac	Glu	suc	mal	gal	man	ara	Suspected Organism
LBI	+ve	Rod	-ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	-ve	*Lactobacillus acidophilus*
LBII	+ve	Rod	-ve	+ve	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	*Lactobacillus casei*
LBIII	+ve	cocci	-ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	*Strep. thermophilus*
LBIV	+ve	cocci	-ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	*Lactococcus lactis*
LBV	+ve	Cocco bacilli	-ve	+ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	*Lactobacillus fermentum*
LBVI	+ve	Cocco bacilli	-ve	+ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve	*Lactobacillus brevis*

Antimicrobial activity of Bacteriocin against *Mycobacterium sp* in milk

The growth pattern of *M. bovis* and *M. tuberculosis* in milk subjected to different concentrations of bacteriocin from the six LAB isolates were evaluated spectrophotometrically and the results presented in Figures 1 and 2 for the single bacteriocin treatment. The different LAB bacteriocins had significant effects ($p \leq 0.05$) on the growth of *M. bovis and M. tuberculosis* in their respective milk cultures.

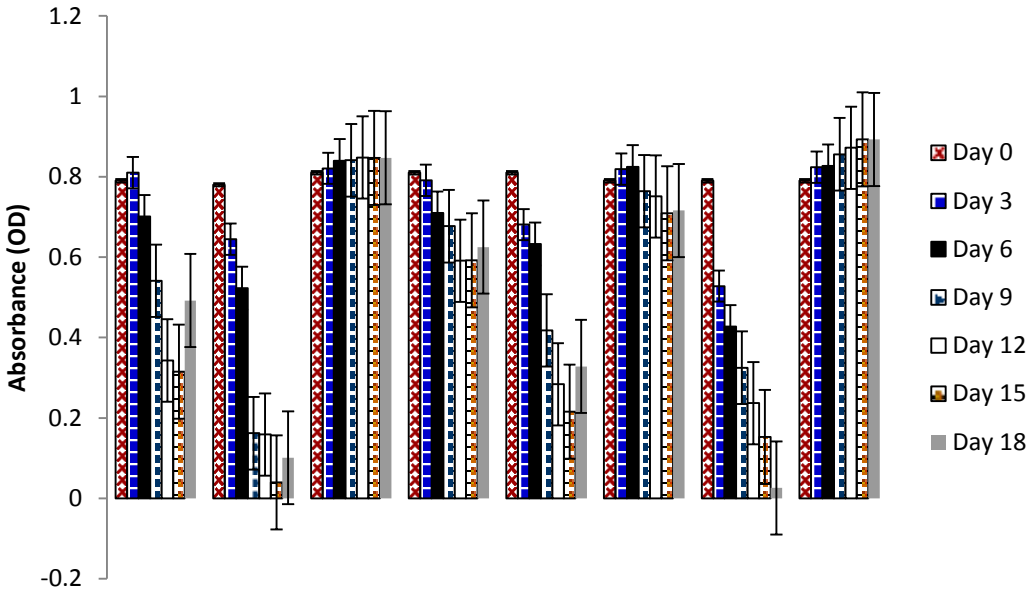

Figure 1: **Growth inhibition of *Mycobacterium bovis* by the individual bacteriocins and bacteriocin Mix in milk**

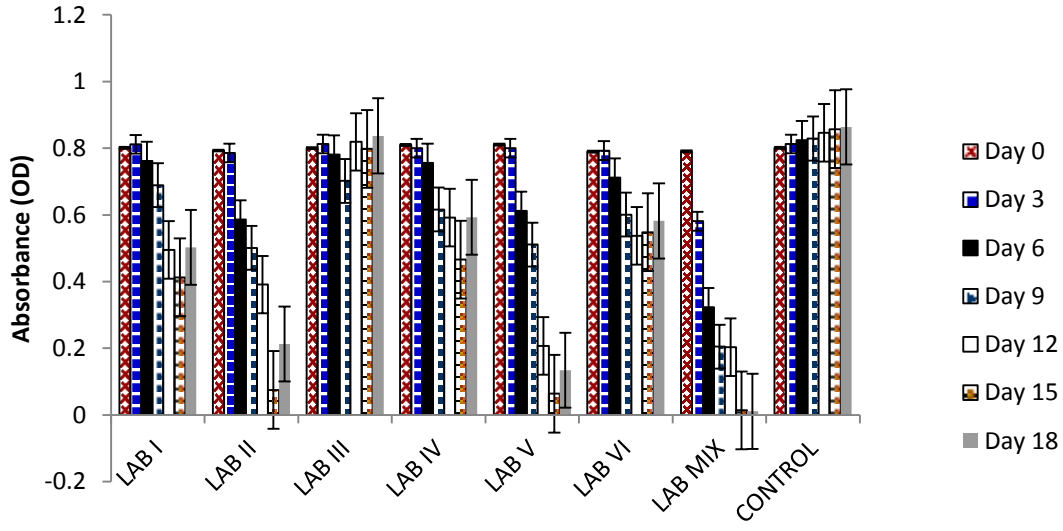

Figure 2: **Growth inhibition of *Mycobacterium tuberculosis* by the individual bacteriocins and bacteriocin Mix in milk**

Antimycobacterial activity of bacteriocin mix

Figure 3 shows the growth pattern of *M. bovis* and *M. tuberculosis* in milk subjected to treatment with a mixture of the bacteriocins from the six LAB isolates. The mixture of bacteriocins had significant effects ($p \leq 0.05$) on the growth of *Mycobacterium* spp in the milk cultures. This can be seen in the decrease in the absorbance from 0.79 on Day 0 to 0.01 on Day 18 (representing 99% growth inhibition) in *M. bovis* and from 0.79 on Day 0 to 0.02 on Day 18 (representing 98% growth inhibition) in *M. tuberculosis.*

A comparison of the effect of individual bacteriocin with the mixture of bacteriocins from the LAB isolates, shows that the bacteriocin mix had a significant ($p \leq 0.05$) growth inhibition than the individual LAB bacteriocins over the 18 day incubation period. This could be seen in the decrease in absorbance from 0.81 on Day 0 to 0.001 on Day 18 represented 99.9% growth inhibiton of the test organisms respectively. The decline in the growth of the *Mycobacterium* spp was steady and depicted an efficient inhibitory activity of the bacteriocins from LAB.

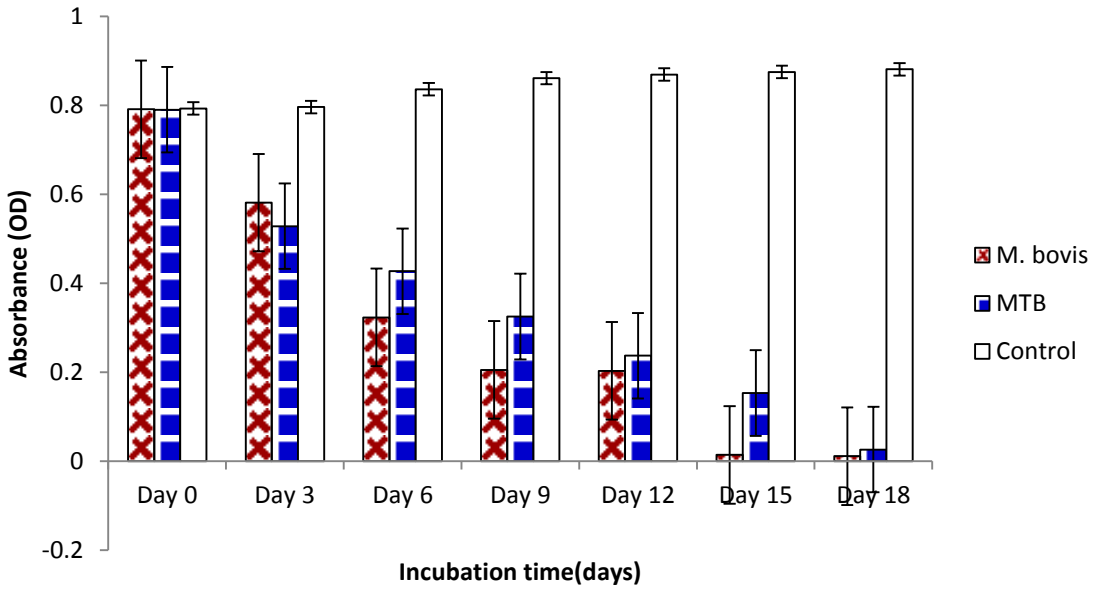

Figure 3: Growth of *M. bovis* and *M. tuberculosis* in the presence of bacteriocin mix in milk

DISCUSSION

Mycobacteria have been noted to be highly resistant to a wide range of antimicrobials including organic acids, and other low molecular weight antimicrobials from LAB. However, there is an increasing hypothesis that mycobacterial inhibition by LAB is possible via the activity of bacteriocins . With respect to the inhibition of *Mycobacteriumsp* by the bacteriocins, it was observed that *L. casei* and *L. fermentum* bacteriocins had higher inhibition reducing the turbidity of MTB from 0.73 to 0.075 and from 0.811 to 0.064 respectively. While for *M. bovis*, the two bacteriocins reduced

the turbidity from 0.78 to 0.04 and 0.811 to 0.216 respectively. *S. thermophilus* bacteriocin had no inhibition against the Mycobacteria.

However, the observation that the mycobacteria growth decreased up to the 15th day and increased on the 18th day is indicative of the fact that individual bacteriocins from the LAB were bacteriostatic in action. This could be because of several factors including bacteriocin dose and degree of purification, physiological state of the cells and experimental conditions thus allowing the resurgence of mycobacteria growth. In a hypothetical attempt to explain this occurrence, the physiology of *M. bovis* shows it as a slow growing microorganism, as it grows by a gradual builds up in its metabolism. Biophysically, the slow rate of cell growth in mycobacteria overrides the effects of the concentration of available chemical substances within such a system, as every concentration has to make a considerable impact based on the contact time with individual cells. It was also observed that the use of the mixture of bacteriocins from the six LAB proved to be more effective in inhibition when compared with the bacteriocin from the individual organisms. These are in line with findings of Sieuwerts et al., about the combinational inhibitory effect of LAB species due to synergistic relationships. The bacteriocin mix inhibited Mycobacteria growth up to the 18th day reducing the turbidity from 0.79 to 0.026 for *M. bovis* and 0.791 to 0.071 for MTB. The activity of the bacteriocin mix could be said to be bacteriocidal. Although the mode of action of bacteriocin against mycobacteria was not part of this study, it could be that the bactericidal activity of the test bacteriocins may be due to the lysis of sensitive cells. Binding of these bacteriocins to the negatively charged cell wall of the sensitive mycobacteria led to release and therefore activation of autolytic enzymes, which under normal conditions are electrostatically bound to these polymers leading to lysis of sensitive cells. Moreover, it has been reported that bacteriocin exert their bactericidal mode of action by destabilization and permeabilization of sensitive bacterial cell membranes. Some class I (lantibiotic) bacteriocins including nisin were shown to have a dual mode of action. They bind with lipid II, preventing adequate cell wall synthesis and leading to cell death. Moreover, nisin can use lipid II as a docking molecule to induce a process of membrane insertion and pore formation that leads to sudden cell death. **[22-29]**

CONCLUSION

Results of this research showed that the bacteriocins inhibited Mycobacteria growth in milk especially when a mixture of bacteriocins was used in combination. This thus supports the potential usefulness of these bacteriocins as food bio preservatives against mycobacteria in dairy products.

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