The effect of metabolites of lactobacillus in fermented milk on the growth of hospital isolates of *E. coli*

Chika Crescence Ogueke*

Department of Food Science and Technology, *Federal University of Technology*, *Owerri*, *Imo State*, *Nigeria*

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Abstract

The effects of metabolites produced by *Lactobacillus* species in fermented milk on hospital *E. coli* isolates were considered. Starter culture as well as *L. bulgaricus* and *L. acidophilus* were used independently to ferment milk. Well in agar diffusion method was used to determine the antagonistic activities on the isolated. The cell free fermented milks inhibited the growth of the isolated to varying degrees, however, the effect of the starter culture fermented milk was higher than that produced by the different *Lactobacillus* species. The bacteriocin produced in the fermented milk inhibited the growth of the test isolated after heat treatment at 100 ºC for 10 min and 121 ºC for 15 min although the antagonistic activity decreased slightly. Fermentation produced lactic acid, hydrogen peroxide and diacetyl with the starter culture fermented milk producing higher amounts of lactic acid and diacetyl, and *L. acidophilus* fermented milk having more hydrogen peroxide. The results indicate that fermented milk, especially starter culture fermented milk, can be administered in cases of diarrhea due to *E. coli*. [Life Science Journal. 2008; 5(1): 90 – 94] (ISSN: 1097 – 8135).

Keywords: fermented milk; *Lactobacillus*; metabolites; anatagonistic; *E. coli*

1 Introduction

Lactobacillus sp. is bacteria that belong to a group generally referred to as lactic acid bacteria. They are important in the food industry for their fermentative ability and their health, and nutritional benefits (Gilliland, 1990). They are common organism usually isolated from plants, fermenting vegetables, diary, meat products and mucosal surfaces of animals (Lindgren and Dobrogosz, 1990). *Lactobacillus* sp. has been found to produce bacteriocin in addition to lactic acid and hydrogen peroxide during their lactic fermentation (Adams and Moss, 1999). Bactericin have been found to inhibit a wide range of bacteria including Gram positive and Gram negative food spoilage and pathogenic bacteria such as *E. coli* (Ogumbanwo *et al*, 2003; Adams and Moss, 1999). Previous studies have centered on the type of bactericin produced (Mataragas *et al*, 2002; Ten Brink *et al*, 1994; Holo *et al*, 1991; Bhunia *et al*, 1988), influence of cultural conditions on the production of bacteriocins (Ogunbanwo *et al*, 2003; Balasubranyam and Varadaraj, 1998; Graciela *et al*, 1995; Daba *et al*, 1993) and antibacterial activity of the purified bacteriocins (Ogunbanwo *et al*, 2006; Sanni *et al*, 1999; Adersson, 1986).

E. coli is an important food pathogen associated with infections which manifest themselves in the form of diarrhea. This is a common disease condition in the developing countries especially amongst children (Adams and Moss, 1999). Due to the misuse and abuse of antibiotics some of strains of this organism have become resistant to common antibiotics employed in the therapy. This poses a serious threat to children in developing countries. However, it is believed that the use of *Lactobacillus* fermented milk and the antimicrobial metabolites therein could solve the problem. This work was therefore undertaken to determine the effect of these metabolites produced in fermented milk on *E. coli*.

2 Materials and Methods

2.1 Source of materials

Skimmed milk and commercial starter culture were

*Corresponding author. Email: oguekejuly10@yahoo.com

obtained from a reputable store in Owerri, Nigeria. The bacterial isolate *E. coli* was obtained from three different hospital in Imo State, Nigeria (Federal Medical Centre (FMC), Owerri, Imo State University Teaching Hospital (ISUTH), Orlu and General Hospital (GH), Owerri. A standard culture *E. coli* NCTC 10418 was used as control. They were subcultured on Plate Count Agar and later transferred to agar slants made from the agar used for their subculturing. They were stored at 4 ºC until required.

2.2 Preparation of fermented milk using commercial starter culture

400 g of skimmed milk was dissolved in 1 L of purified water (Purite R050 reverse osmosis and ion exchange unit). The milk solution was then heated to 80 ºC on a water bath (Galenkamp, England) for 30 min. The heated milk was cooled to 43 ºC and 5 g of the starter culture was added to the milk, stirred and incubated at 43 ºC for 5 h. After the fermentation the temperature was reduced to 20 ºC and then to 5 ºC. It was stored at that temperature until further use.

2.3 Isolation and characterization of *Lactobacillus* sp.

Lactobacillus bulgaricus (*L. bulgaricus*) and *L. acidophilus* were isolated from the fermented milk using MRS agar (Oxoid CM361). They were subcultured into MRS broth at 30 ºC for 24 h before use. Characterization of isolated was carried out by using macroscopic, microscopic, biochemical tests as well as employing the API 50 CH strips for *Lactobacillus* sp. (API System Biomereux SA, France).

2.4 Fermentation of milk using *Lactobacillus* sp.

The isolated *L. bulgaricus* and *L. acidophilus* were used independently to ferment skimmed milk as was conducted with the commercial starter culture. Standardized broth cultures of the bacteria (1.0×10^7 cfu/ml) were used to carry out the fermentation. After the fermentation they were also stored at 5 ºC until required.

2.5 Quantitative determination of lactic acid, hydrogen peroxide and diacetyl in fermented milk

25 ml of cell free fermented milks were titrated with 0.1 N NaOH. Three drops of phenolphthalein were added as indicator. Titration was carried out until a pink colour appeared. Each ml of 0.1 N was taken to be equivalent to 90.08 mg of lactic acid.

For hydrogen peroxide 20 ml of diluted H_2SO_4 was added to 25 ml of the cell free fermented milk and titrate with 0.1 N potassium permanganate. Titration was carried out until decolourization. Each ml of 0.1 N potassium permanganate was taken to be equivalent to 170 mg of H_2O_2.

Also for diacetyl 25 ml of cell free fermented milk was put in a conical flask and 7.5 ml of hydroxylamine solution was used for the residual titration. This was titrated with 0.1 N HCl to a green-yellowish end point using bromophenol blue as indicator. Each ml of 0.1 N HCl was equivalent to 21.5 mg of diacetyl.

2.6 Preliminary treatment of fermented milk before use for analysis

The already fermented milks were centrifuged at 2500 rpm for 10 min in a bench-top centrifuge to remove microbial cells and other suspended solids to obtain cell free fermented milk. The culture supernatant was concentrated with a rotary evaporator until 50% of the original volume was left. 50 ml of the cell free fermented milk was treated with 5 mg/ml catalase (C-100 bovine liver, Sigma) to eliminate the inhibitory activity of H_2O_2 (Daba *et al*, 1991). The pH was then adjusted to 7 with NaOH. This was used to determine the effect of the bacteriocins on the isolated at neutral pH.

Two 50 ml volumes were also taken. The inhibitory activity of H_2O_2 was eliminated and pH adjusted 7 as above. Each was then heated to 100 ºC for 10 min and 121 ºC for 15 min respectively. These were used to determine the effects of temperature on the activities of the bacteriocins.

2.7 Determination of the antagonistic activity of the fermented milks on the test isolates

The *E. coli* isolated plated out on Plate Count Agar (Oxoid CM463). This was done by transferring 1.0 ml of the standardized broth cultures (1.0×10^7 cfu/ml) into sterile petridishes and pouring about 15 ml of molten agar. Using a sterile cork borer the holes (5 mm each) were made in the agar plates. Equal volumes of the cell free fermented milks were transferred into the holes using Pasteur pipette. Two plates were used for each type of fermented and treated milk. The plates were incubated at 37 ºC for 24 h. At the end of incubation the zones of inhibition produced were measured. Each of the fermented and treated milks were also tested on each *E. coli* isolate.

2.8 Analysis of data

The data obtained were analyzed statistically using Analysis of Variance. The means were separated using Fisher's Least Significant Difference.

3 Results

The results obtained from the study indicated that the

fermented milks inhibited the growth of the isolates to varying degrees. However, the inhibitory effect of the milk fermented with the starter culture was more than that produced by the milks fermented by the different *Lactobacillus* sp.

At neutral pH (Table 1) the zones of growth inhibition produced by the different fermented milks decreased although the inhibitory effect of starter culture fermented milk was still higher than others.

Heat treatment of the various fermented milks at 100 ºC for 10 min and 121 ºC for 15 min slightly reduced the inhibitory effects of the fermented milks (Tables 2 and 3), but still maintained the same trend as was found in Tables 1 and 4.

Statistical analysis of the data obtained revealed that in general, at neutral pH and after the heat treatments there was significant difference ($P = 0.05$) in the effects of each fermented milk on the isolates. However, results in Table 4 indicated that the effects of each fermented milk on *E. coli* NCTC 10418 was significantly different ($P = 0.05$) from the effects on the other isolates.

Results of the levels of lactic acid, H_2O_2 and diacetyl in the fermented milks (Table 5) indicated that the starter culture fermented milk produced more lactic acid (1.72 g/L) and diacetyl (0.54 g/L) while the milk fermented with *L. acidophilus* produced the highest amount of H_2O_2 (0.21 g/L).

4 Discussion

Results obtained from the study showed that the fermented milks inhibited the test hospital isolates and the standard culture (*E. coli* NCTC 10418) to varying degrees. Other worked have also found that metabolites produced by *Lactobacillus* sp. inhibited bacteria they used for their study (Sann *et al*, 1999; Ogunbanwo *et al*, 2003; Ogunnbanwo *et al*, 2006; Bredholt *et al*, 1999; De Martins and Franco, 1998). However, the

Table 1. The effect of neutral pH on the antagonistic activity of bacteriocins produced by *Lactobacillus* sp. in fermented milk on *E. coli*

Type of fermented milk	Zone of growth inhibition (mm)			
	E.coli FMC	*E.coli* GH	*E.coli* ISUTH	*E.coli* NCTC10418
Starter culture fermentation	$19.3 \pm 0.4^{a,b}$	18.9 ± 0.31^{b}	19.8 ± 0.34^{a}	20.6 ± 0.18^{a}
L. bulgaricus fermentation	10.3 ± 0.82^{b}	11.5 ± 0.18^{a}	$11.1 \pm 0.26^{a,b}$	11.4 ± 0.11^{a}
L. acidophilus fermentation	11.3 ± 0.35^{a}	11.4 ± 0.71^{a}	11.7 ± 0.41^{a}	11.6 ± 0.30^{a}

Values are means of triplicate readings ± standard deviation. ^{a,b} values with different superscript on the same row are significantly different ($P = 0.05$).

Table 2. The effect of heat at 100 ºC for 10 min on the antagonistic activity of bacteriocins produced by *Lactobacillus* sp. in fermented milk on *E. coli*

Type of fermented milk	Zone of growth inhibition (mm)			
	E.coli FMC	*E.coli* GH	*E.coli* ISUTH	*E.coli* NCTC10418
Starter culture fermentation	$18.7 \pm 0.50^{a,b}$	18.2 ± 0.14^{b}	19.1 ± 0.27^{a}	19.5 ± 0.33^{a}
L. bulgaricus fermentation	10.1 ± 0.64^{b}	10.8 ± 0.39^{a}	$10.3 \pm 0.21^{a,b}$	10.8 ± 0.25^{a}
L. acidophilus fermentation	10.5 ± 0.08^{a}	10.2 ± 0.40^{b}	$10.6 \pm 0.22^{a,b}$	11.2 ± 0.71^{a}

Values are means of triplicate readings ± standard deviation. ^{a,b} values with different superscript on the same row are significantly different ($P = 0.05$).

Table 3. The effect of heat at 121 ºC for 15 min on the antagonistic activity of bacteriocins produced by *Lactobacillus* sp. in fermented milk on *E. coli*

Type of fermented milk	Zone of growth inhibition (mm)			
	E.coli FMC	*E.coli* GH	*E.coli* ISUTH	*E.coli* NCTC10418
Starter culture fermentation	18.4 ± 0.63^{a}	17.5 ± 0.48^{b}	18.5 ± 0.31^{a}	18.6 ± 0.13^{a}
L. bulgaricus fermentation	10.2 ± 0.22^{a}	10.4 ± 1.01^{a}	9.6 ± 0.84^{b}	10.1 ± 0.57^{a}
L. acidophilus fermentation	10.1 ± 0.69^{a}	10.2 ± 0.73^{a}	10.3 ± 0.30^{a}	10.6 ± 0.88^{a}

Values are means of triplicate readings ± standard deviation. ^{a,b} values with different superscript on the same row are significantly different ($P = 0.05$).

Table 4. Antagonistic activity of metabolites produced by *Lactobacillus* sp. in fermented milk on *E. coli*

Type of fermented milk	Zone of growth inhibition (mm)			
	E.coli FMC	*E.coli* GH	*E.coli* ISUTH	*E.coli* NCTC10418
Starter culture fermentation	25.2 ± 0.16^{b}	28.3 ± 0.37^{a}	24.5 ± 0.21^{b}	29.7 ± 0.62^{a}
L. bulgaricus fermentation	18.3 ± 0.26^{a}	18.1 ± 0.41^{a}	$18.6 \pm 0.12^{a,b}$	20.2 ± 0.31^{b}
L. acidophilus fermentation	18.5 ± 0.11^{a}	$18.7 \pm 0.08^{a,b}$	18.4 ± 0.51^{a}	19.3 ± 0.22^{b}

Values are means of triplicate readings ± standard deviation. ^{a,b} values with different superscript on the same row are significantly different ($P = 0.05$).

Table 5. The level of lactic acid, hydrogen peroxide and diacetyl produced in fermented milk by the *Lactobacillus* sp.

Type of fermented milk	Zone of growth inhibition (mm)		
	Lactic acid (g/L)	H_2O_2 (g/L)	Diacetyl (g/L)
Starter culture fermentation	1.72	0.12	0.54
L. bulgaricus fermentation	1.28	0.18	0.40
L. acidophilus fermentation	1.33	0.21	0.33

Values are means of triplicate readings.

level of inhibitory exhibited by the commercial starter culture fermented milk was higher than by the different *Lactobacillus* sp.

The observed higher levels of inhibition could be attributed to the higher amounts of antibacterial metabolites produced by the starter culture. They produced higher quantities of lactic acid (1.72 g/L) and it is supposed that they also produced higher levels of bacteriocins. The higher levels of inhibition observed in Tables 1, 2 and 3 is an indication that higher amounts of bacteriocins were produced by the starter culture. The higher amounts of bacteriocins and lactic acid produced could be attributed to the cultural conditions and composition which favoured their production. Starter cultures are composed of *Streptococcus thermophilus*, *L. bulgaricus* and *L. acidophilus*. These organisms when used in milk fermentation maintain a relationship referred to as proto co-operation which means that they have a mutually favourable interaction but are not completely interdependent. They will grow on their own in milk but will grow and acidify the product faster when present together (Adams and Moss, 1999). The growth of the streptococcus in milk is limited by the availability of peptides and free amino acids which are present in relatively low concentrations (about 50 mg/kg). However, the lactobacilli are slightly proteolytic and liberates small amounts of these particularly valine, which stimulates streptococcal growth. In its turn the streptococcus produces formate, pyruvate and CO_2 all of which stimulate lactobacilli (Adams and Moss, 1999). Since *L. bulgaricus* and *L. acidophilus* were used independently in the fermentation it could be that they lacked these compounds which stimulate their growth and probably bacteriocin production. In fact *L. bulgaricus* tends to grow poorly in milk with low levels of formate, forming elongated, multinucleate cells (Adams and Moss, 1999). Several workers have reported that cultural conditions and composition affect the level of bacteriocins produced by *Lactobacillus* sp. (Biswas *et al*, 1991; Daba *et al*, 1993; Sanni *et al*, 1999; Ogunbanwo *et al*, 2003).

At neutral pH and without any heat treatment (Table 1) it was observed that the inhibitory effects reduced considerable. This could be assumed to be the actual inhibitory effect of the bacteriocins since the effect of pH and H_2O_2 have been eliminated. The higher values in Table 4 could be the result of the combined effect of the effect of the lactic acid itself. Usually the effect of two or more factors inhibitory to bacterial growth elicits an inhibitory action greater than either of the different factors (synergism). The higher inhibitory effect observed with the starter culture fermented milk was also attributed to cultural conditions and composition which favoured production of more antibacterial metabolites.

Heat treatment of the fermented milks slightly reduced their antibacterial effects on the isolates; reduction increased with temperature and exposure time (Tables 2 and 3). It could be that as the temperature increased the bacteriocins were destroyed. However, the bacteriocins could be assumed to be heat stable since the reductions were negligible. Temperature stability is an important factor to consider if the bacteriocins are to be used as bipreservatives. Ogunbanwo *et al* (2006) reported that their bacteriocins were heat stable at 100 ºC for 10 min and decreased in antagonistic activity when exposed to a temperature of 121 ºC for 10 min. Andersson (1986) also reported that heat treatment at 121 ºC for 15 min reduced the activity of bacteriocins produced by *Lactobacilus* sp.

The high level of inhibition exhibited against *E. coli*

is of significance. Such fermented milks especially those fermented by the starter culture, could be administered to children and others alike in cases of diarrhea or such infections. That the bacteriocins operate in an acidic environment is an indication that their activity would not be affected by the gastric juice in the stomach. In fact the low pH will increase their effects as observed in Table 4. Thus they can reach the intestines and effect their antibacterial action. Yoghurt has been shown to have a strong inhibitory effect on the growth of coliform bacteria in the stomach and duodenum of piglets (Adams and Moss, 1999).

In conclusion fermentation of milk with starter cultures as it is commonly practiced produced more inhibitory effects on the *E. coli* isolates, an indication that they produced more antibacterial metabolites. Therefore administration of starter culture fermented milk in cases of diarrhea could bring relief especially in children. This is more so because fermented milk is a widely accepted product amongst children and even adults.

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