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Addition of Inulin and Manganese Chloride Increased Antibacterial Metabolites in Yoghurt

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Abstract: The effect of the addition of inulin and manganese chloride ($MnCl_2$) to milk before fermentation on the amount of antibacterial metabolites produced in fermented milk was studied. Normal and modified fermented milks were produced and the lactic acid and hydrogen peroxide (H_2O_2) contents were determined using standard analytical procedures. The well in agar diffusion method was used to determine the antibacterial effects of the cell free fermented milks on *Staphylococcus aureus* and *E. coli*. Results obtained showed that addition of the compounds increased the amount of lactic acid and H_2O_2 in the modified milk. Highest amounts of lactic acid (6197.50 mg/L) and H_2O_2 (42.83 mg/L) were obtained in the sample containing highest amounts of inulin (7 g/L) and $MnCl_2$ (2 mg/L). The largest zones of growth inhibition for *S. aureus* and *E. coli* were obtained in samples containing 5 g/L inulin, 2 mg/L $MnCl_2$ and 7 g/L inulin, 2 mg/L $MnCl_2$, respectively. However, larger zone of growth inhibition were generally observed with *E. coli*. The inclusion of these substances significantly increased the amounts of the antibacterial metabolites and increased the inhibitory effect on *S. aureus* and *E. coli*. Therefore addition of such compounds to milk before fermentation should be encouraged to improve the probiotic and health potentials of fermented milk.

Key words: Antibacterial metabolites, inulin, manganese chloride, inhibition, bacterial isolates,

INTRODUCTION

Lactic Acid Bacteria (LAB) are the most commonly used microbes in the production of various fermented foods such as dairy, beverages, meat and vegetables. They have been used in the food industry for many years because they are able to convert sugars to lactic acid. This provides the characteristic sour taste of fermented dairy products such as yoghurt and also lowers pH thereby making the environment unfavourable to spoilage microorganisms, thus creating possible health benefits by preventing gastrointestinal infections (Rodriquez *et al*., 2000). LAB strains of the genera Lactobacillus and Bifidobacterium are the most widely used probiotic bacteria (Marteau *et al*., 2001).

LAB are used as natural or selected starters in food fermentations in which they perform acidification of products and addition of flavours. They produce various metabolites in the products which protect the products from spoilage microorganisms and pathogens. These include organic acids, hydrogen peroxide (H_2O_2) and diacetyl (Messens and De Vugst, 2002), antifungal compounds (Corsetti *et al*., 1996), phenullatic acid (Lavermicocca *et al*., 2000) and bacteriocins (De Vugst and Vandamme, 1994).

Some researchers have shown that modification of cultural environment increases bacteriocin production by LAB (Ogunbanwo *et al*., 2003; Barasubramanyam and Varadaraj, 1998; Graciela *et al*., 1995) and also manganese chloride ($MnCl_2$) and oligosaccharides (inulin) greatly enhance the growth of these bacteria (Adams and Moss, 1999). It is therefore believed that addition of these compounds to milk during yoghurt production will ultimately increase the production of these metabolites (lactic acid, H_2O_2) that will confer some protection on the final product as well as increase bacteriocin production and improve the health benefits that could be derived from the product.

Hence this work was undertaken to determine the effect of different levels of $MnCl_2$ and inulin on the level of lactic acid and H_2O_2 produced during yoghurt production and the inhibitory effect of the modified fermented milk on some bacterial isolates.

MATERIALS AND METHODS

Source of materials: Skimmed milk (Carnco Foods Ltd.) and starter culture (Yogomaster, Lyo-sah Inc. Canada) were obtained from reputable stores in Owerri, Nigeria while inulin and $MnCl_2$ were obtained from reputable stores in Lagos, Nigeria. The bacterial isolates *E. coli* and *Staphylococcus aureus* were obtained from the medical laboratory of Federal Medical Centre, Owerri, Nigeria. *E. coli* was subcultured on Plate Count Agar while *S. aureus* was subcultured on Baird Parker Agar and were later transferred to agar slants made from the agar used for their sub-culturing. They were stored at 4°C until required.

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Preparation of fermented milk: Four hundred grams of skimmed milk was dissolved in 1000 mL of purified water (Purite R050 reverse osmosis and ion exchange unit) and heated at 80°C for 30 min. This was cooled to 43°C and 5 g of the starter culture comprising *Lactobacillus bulgaricus*, *Streptococcus thermophilus* and *L. acidophilus* was added, stirred and allowed to ferment at 43°C for 6h. At the end of fermentation the yoghurt curd was passed through a fine screen to remove any nodule, thus smooth consistent yoghurt was produced. The temperature was reduced to 20°C and then to 5°C and stored at that temperature until required.

Preparation of modified fermented milk: This was prepared as above except that different quantities of inulin and $MnCl_2$ were added to the milk before the addition of the starter cultures for fermentation. The table below shows the quantities of the compounds added.

Preparation of cell free fermented milk: The normal and modified fermented milks were retrieved from the refrigerator and centrifuged with Hattich Universal centrifuge at the speed of 2500 rpm for 30 min to remove microbial cells and other suspended solids to obtain cell free fermented milk. The cell free fermented milk was then used for the study.

Quantitative determination of lactic acid and H_2O_2 in fermented milk: Twenty five milliliters of cell free fermented milks were titrated with 0.1N NaOH. Three drops of phenolphthalein were added as indicator. Titration was carried out until a pink colour appeared. Each mL of 0.1N NaOH was taken to be equivalent to 90.08 mg of lactic acid (Ogueke and Nwagwu, 2007).
For H_2O_2, 20 mL of dilute H_2SO_4 was added to 25 mL of the cell free fermented milk and titrated with 0.1N potassium permanganate solution. Titration was carried out until decolourization. Each mL of 0.1N potassium permanganate was taken to be equivalent to 1.70 mg of H_2O_2 (Ogueke, 2008).

Determination of inhibitory effects of bacteriocin in cell free fermented milk samples: Fifty milliliters of the cell free fermented milk samples were 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 to eliminate the inhibitory effect of lactic acid. Nutrient agar plates seeded with the test isolates (*S. aureus* and *E. coli*) were prepared according to the methods described by Ogueke (2008). Four holes measuring 5.0 mm in diameter were made in each plate using a sterile cork borer. Equal volumes of the cell free fermented milks were transferred into the holes using Pasteur pipette. Three plates seeded with each of the isolates were used for each type of fermented milk sample. The plates were incubated at 37°C for 24h. At the end of incubation the zones of growth inhibition produced were measured.
All analyses were carried out in triplicate.

Sensory evaluation of products: Sensory evaluation of the products was conducted using the 9 point hedonic scale as described by Iwe (2002). The attributes analyzed for were taste, texture, colour and general acceptability of the products.

Analysis of data: The data obtained from the study were analyzed using Analysis of Variance (ANOVA). The means were separated using Fisher's Least Significant Difference (LSD). Microsoft Excel was used for the computation.

RESULTS AND DISCUSSION

Table 2 shows the amount of lactic and H_2O_2 produced in the various fermented milk samples by the starter cultures. For lactic acid the largest amount was produced in sample D (6197.50 mg/L). All the other samples had higher values than the normal fermented milk which had a value of 3855.42 mg/L of fermented milk. Sample D also had the highest value for H_2O_2 with a value of 42.83 mg/L of fermented milk. The normal fermented milk had the least value (14.45 mg/L).
The increased levels of these metabolites could be attributed to the addition of inulin and $MnCl_2$. Sample D which produced the largest amount of lactic acid (6197.50 mg/L) and H_2O_2 (42.83 mg/L) had the highest quantities of inulin and $MnCl_2$ added. 7g/L of inulin and 2mg/L of $MnCl_2$ were added to the milk before fermentation. Statistical analysis of the values still buttressed the fact. The values for lactic acid and H_2O_2 for sample D were significantly different from all other values obtained in the study. The values from all the modified fermented milk samples were higher and also significantly different from the values obtained from the normal fermented milk. These bacteria (*Lactobacillus* sp.) are known to breakdown oligosaccharides (such as inulin) which they utilize for growth and produce short chain fatty acids (SCFAs) and lactic acid. Such SCFAs have been shown in some studies (Scholz-Ahrens *et al*., 2001; Shulz *et al*., 1993; Scharrer and Lutz, 1992) to improve the metabolic absorption of various ions such as Fe, Ca and Mg. $MnCl_2$ is also known to stimulate their growth (Adams and Moss, 1999).
With regards to the amount of H_2O_2 that should be produced in fermented milk, Marteau and Rambaud (1996) suggested that it should not exceed the range from 20.4 mg/L-81.6 mg/L, which these workers said would not compromise gut health. The results obtained in this work show that the amounts of H_2O_2 produced in the modified fermented milks were within the range. The H_2O_2 values obtained ranged from 36.21 mg/L to 42.83 mg/L. H_2O_2 is also known to potentiate the

Table 1: Quantity of components added to milk before fermentation

Sample	Inulin (g/L)	$MnCl_2$ (mg/L)
A	3	2
B	3	1
C	5	1
D	7	2
E	5	2

Table 2: Lactic acid and H_2O_2 produced in fermented milk samples (mg/L)

Sample	Lactic acid	H_2O_2
A	4008.86^{b}	41.82^{d}
B	5494.88^{c}	36.21^{b}
C	5765.12^{d}	37.91^{c}
D	6197.50^{f}	42.83^{e}
E	6071.39^{e}	42.16^{d}
Normal fermented milk	3855.42^{a}	14.45^{a}
LSD	0.86	0.58

a,b,c..., Values on the same column with different superscript are significantly different ($p<0.05$)

lactoperoxidase antimicrobial system in milk (Adams and Moss, 1999). This will increase the antibacterial properties of the fermented milk, thus increasing the benefits derivable from consumption of such fermented milks.

Table 3 shows the zones of growth inhibition produced by the various fermented milk samples on the test isolates. Since the inhibitory effects of lactic acid and H_2O_2 in the fermented milk samples were neutralized before using them for the test, the zones of growth inhibition observed could be attributed to the effects of the bacteriocins in the fermented milk samples. The results revealed that the added compounds apparently stimulated the growth of the LAB which presumably resulted in increased bacteriocin production and ultimately increased inhibition of the bacterial isolates. The zones of growth inhibition produced on *E. coli* were generally higher than those produced on *S. aureus*. Statistical analysis indicated that all the values obtained were significantly higher than those obtained on *S. aureus*, except sample C which contained 5g/L inulin and 1mg/L $MnCl_2$.It may be that *E. coli* was more susceptible to the type of bacteriocin (s) produced by the starter cultures. *Lactobacillus acidophilus* strains are known to produce lactacin B (Barefoot and Klaenhammer, 1983) while *S. aureus* is killed by lysostaphin (Bastos *et al*., 2010). Some studies (Bogovic-Matijasic *et al*., 1998) have shown that the bacteriocins acidocin LF221 A and acidocin LF221 B produced by *L. acidophilus* inhibit the growth of *S. aureus* to a high degree. Other *Lactobacillus* species have been found to inhibit both *S. aureus* and *E. coli* (Jamuna *et al*., 2005). The observed largest zone of growth inhibition on *S. aureus* was 26.0 mm while that of *E. coli* was 26.4 mm. These were produced by samples E and D respectively. The values were significantly

Table 3: Zones of growth inhibition produced on the isolates by the fermented milk samples (mm)

Sample	*S. aureus*	*E. coli*	LSD
A	$^{1}23.7^{b}$	$^{2}26.2^{e}$	0.33
B	$^{1}23.8^{b}$	$^{2}25.3^{d}$	0.10
C	$^{1}24.4^{d}$	$^{1}24.6^{c}$	0.95
D	$^{1}24.2^{c}$	$^{2}26.4^{f}$	1.05
E	$^{1}26.0^{e}$	$^{2}24.0^{b}$	0.17
Normal fermented milk	$^{1}16.9^{a}$	$^{2}17.8^{a}$	0.33
LSD	0.12	0.06	

a,b,c..... Values on the same column with different superscript are significantly different ($p<0.05$)

1,2,3..... Values on the same row with different superscript are significantly different ($p<0.05$)

Table 4: Mean sensory attributes of fermented milk products

Samples	Taste	Texture	Colour	General acceptability
A	6.9^{b}	6.4^{c}	8.2^{a}	6.5^{b}
B	6.7^{b}	$7.0^{b,c}$	8.2^{a}	6.6^{b}
C	6.8^{b}	$7.1^{b,c}$	8.3^{a}	6.5^{b}
D	7.9^{a}	$7.7^{a,b}$	8.3^{a}	7.9^{a}
E	7.1^{b}	7.2^{b}	8.2^{a}	6.9^{b}
NFM	8.4^{a}	8.2^{a}	8.6^{a}	8.3^{a}
LSD	0.81	0.75	0.94	0.97

a,b,c...., Values on the same column with different superscript are significantly different ($p<0.05$). NFM: Normal fermented milk

different from the values obtained from the other samples. It is interesting to note that samples D and E contain 2 mg/L of $MnCl_2$. All the values obtained from modified fermented milks were generally higher than those obtained from the normal fermented milk (16.9 mm and 17.8 mm, respectively).

The results were expected since bacteriocin production is directly associated to growth rate, thus composition of the growth media would influence rate of production and amount of bacteriocin produced. Higher amounts of bacteriocin can be produced in media containing different carbohydrate sources (Ogunbanwo *et al*., 2003; Matsuaki *et al*., 1996). Inulin being a carbohydrate (oligosaccharide) could have influenced higher production of bacteriocin, hence larger zones of growth inhibition as observed in the modified fermented milk samples.

The results obtained in this study are of great significance as the two isolates are common causes of food related diseases especially in developing countries (Adams and Moss, 1999). Such modified fermented milks could be administered to children in cases of diarrhea due to *E. coli*.

Table 4 shows the values obtained for the various sensory attributes of the products. The taste and general acceptability of the modified fermented milk products differed significantly ($p<0.05$) with the normal fermented milk except for sample D (which contained 7 g/L inulin and 2 mg/L $MnCl_2$). Scores obtained for colour did not differ significantly ($p>0.05$) in all the fermented milk products. Therefore the level of addition of inulin and

$MnCl_2$ in sample D will still be acceptable to consumers and also stimulate the production of the metabolites by the starters, thus allowing the consumer to derive the important health benefits from the product. However, the scores obtained from the modified fermented milk samples differed significantly ($p>0.05$) from the normal fermented milk in the texture attribute.

Conclusion: Fermented milk is a product that is gaining more popularity because of its health promoting potentials through the ingestion of probiotics. Compounds believed to stimulate the increased production of antibacterial metabolites in milk by the milk fermenting starter cultures were added to milk before fermentation. After fermentation higher amounts of lactic acid and H_2O_2 were produced in the modified fermented milk samples. The modified fermented milk samples also exhibited greater inhibitory effects on *S. aureus* and *E. coli* than normal fermented milk, although the inhibitory effect was more on *E. coli*. Therefore the addition of these compounds to milk before fermentation with starter cultures should be encouraged to improve the probiotic and health potentials of fermented milk.

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