

Full Length Research Paper

Study on the antibacterial properties of probiotic bacteria isolated from human breast milk

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Accepted 21 September, 2017

Human breast milk is said to be rich in various bioactive compounds which promote the maturation of immune system as well as developing body's defense against infections in infants. Breast milk was obtained from six different volunteer breastfeeding mothers, grouped into sample [A, B, C, D, E and F]. Result showed that sample D had the highest LAB count of 1.6×10^8 cfu/ml ($P > 0.05$) while sample A had the least LAB count of 1.3×10^6 cfu/ml ($P > 0.05$). The isolates from the breast milk samples were characterized as *Lactobacillus plantarum* and *Lactobacillus pentosus* and were observed as potential probiotics. These bacteria were facultative anaerobic, gram positive, catalase negative and non-endospore forming. They showed tolerance against 0.3% bile concentration and 1-10% NaCl. Sugar fermentation patterns of the isolated bacteria also greatly varied. The LAB isolates; *L. plantarum* and *L. pentosus* showed a great antimicrobial effect on all the indicator organisms. *Klebsiella* sp. had the highest ($P < 0.05$) susceptibility of 16 ± 1.41 mm and 14 ± 8.48 mm with *L. plantarum* and *L. pentosus*, respectively while *Escherichia coli* had a greater resistance ($P < 0.05$) of 11 ± 0.7 mm and 6 ± 2.82 mm with *L. plantarum* and *L. pentosus*, respectively. The addition of breast milk probiotics to infant formulas could be a new alternative to mimic some of the functional effects of human milk in children who are not breastfed.

Key words: Breast milk, lactic acid bacteria, API50CHL, antimicrobial, *Lactobacillus plantarum*, *Lactobacillus pentosus*.

INTRODUCTION

Probiotics are live microorganisms which are defined by the World Health Organization and Food and Agricultural Organization (FAO/WHO, 2001) as live microorganisms whose administration in adequate amount to the body is able to confer a health beneficial effect on the host (Dunne et al., 2001). The most common types of microbes which are used as probiotics are lactic acid bacteria (LAB) and Bifidobacteria. A number of genera within *Firmicutes* phylum like *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Carnobacterium*, *Enterococcus*, *Streptococcus*, *Tetragenococcus*, *Oenococcus*, *Pediococcus*, *Weissella*, *Melissococcus*, *Vagococcus* constitute lactic acid bacteria (Jay, 2000; Holzapfel et al.,

1998). LABs are Gram-positive bacteria (Fooks et al., 1999) able to ferment carbohydrates into lactic acid and energy (Jay, 2000). Some LAB differ in their metabolic pathway, for example homofermentative bacteria like *Lactococcus* and *Streptococcus* produce two lactate molecules from one glucose molecule while heterofermentative bacteria like *Leuconostoc* is able to convert one molecule of glucose into ethanol, lactate and carbon dioxide (Fooks et al., 1999; Jay, 2000).

Furthermore, lactic acid bacteria yield some organic compounds that contribute to the aroma as well as flavor of the fermented products (Fooks et al., 1999). Human milk is a complex biological fluid that is species-specific

and completely fulfills both nutritional and microbiological requirements of the new born. Breast milk boosts up immune system and builds body defense against various infectious diseases which makes it superior to other food supplements for infants. Various bioactive compounds like immunoglobulins, lysozyme, antimicrobial acids, oligosaccharides, glycoproteins, for example lactoferrin, polyamines, immune cells and bioactive peptides present in breast milk, are responsible for its anti-infective effect (Fooks et al., 1999). These bioactive compounds of human milk play a major role in the regulation of the anti-inflammatory system. Due to immunomodulatory action of human milk, the incidence as well as severity of various infectious diseases like tetanus, poliomyelitis and diphtheria is lesser in breast-fed infants than those fed with other food formulae (Holzapfel et al., 1998). This work is aimed at determining the Antibacterial Properties of Probiotic bacterial Isolated from Human Breast Milk.

MATERIALS AND METHODS

Sample collection

Samples were collected from six (6) different mothers who voluntarily donated their breast milk in Owerri under aseptic conditions. Mothers declared to be in good, healthy condition, having had normal and full-term pregnancy without infant or maternal problems were enrolled in the study. The mammary areola and breast skin were carefully cleaned with soap and rinsed several times with sterile water. First 500 µl of breast milk were discarded followed by the release of 500-700 µl, collected in sterile carriers. Samples were immediately cooled to 5°C and stored at -20°C.

Isolation of lactic acid bacteria

Samples were serially diluted up to 10^{-3} dilutions using sterile peptone water. 1 ml aliquots of dilutions were plated into Man, Rogosa and Sharpe agar (MRSA) (pH 6.2 and pH5.5), agar (pH 6.5). The plates were incubated at 37°C for 72 h under aerobic conditions. The media were specialized to isolate and enumerate the *Lactobacilli* species. One to three bacterial colonies were randomly selected and inoculated with streak plate technique on duplicate MRSA agar (Fooks et al., 1999). They were sub-cultured in MRS broth and again streaked onto MRSA to get pure colonies.

Morphological identification of the isolate

Isolates were tested for Gram-staining reaction, catalase activity and cell morphology. All Gram positive and catalase negative rods were tested for growth in MRS

broth at 15°C and production of gas from glucose. The tests were done according to the instructions of the manufacturer and the results were read after incubation of strains at 37°C for 3 days.

Identification of lactic acid bacteria

It was carried out using the API50CHI kit which contains 49 different sugar tests. The isolates were diluted in 9 ml of normal saline and mixed with the already prepared bromchresol purple which came with the kit. Later the mixture was added, mineral oil was then placed to create an anaerobic condition, incubation took place within 24 to 48 h; the result was generated from the API data base.

Antimicrobial activity

Three Indicator pathogenic microorganisms (*Escherichia coli*, *Staphylococcus aureus*, *Salmonella* sp., *Shigella* sp. and *Klebsiella* sp.) were collected from the Microbiology unit, Federal Medical Centre, Owerri, Imo State, Nigeria. The inocula of these isolates were prepared according to Mac-Farland standard of 0.5 concentrations as described by Cheesbrough (2004). The indicator pathogens were streaked on the MRS agar plate and a disc diffusion method were used to test the lactobacillus probiotics against the pathogens. Plates were incubated to grow cultures for 24 h at 37°C. Inhibition zone diameters surrounding the spotted isolates were measured. Isolates, which gave an inhibition zone bigger than 1 mm, were determined to have antimicrobial activity. All assays were performed in duplicate and the results presented were the means of duplicate trials.

Statistical analysis

All experimental set ups were carried out in triplicates. Results were presented as mean and standard deviations of triplicate determinations.

RESULTS

Bacteria isolated from human breast milk which grew on MRS agar media having a small circular, white-creamy colour, convex and non-transparent colonies were identified as *Lactobacillus* spp. by observing their colony morphology, physiological as well as biochemical characteristics using the API kit. Microscopically, they were Gram-positive, rod shaped, non-motile, catalase negative, and lacked endospores. In the study, the *Lactobacillus* specie identified were *L. plantarum* and *L. pentosus*. The biochemical characterization using the API50CHL kit is shown in Table 1.

Table 1. API carbohydrate utilization profile

| Isolates | Glycerol | Erythritol | D-arabinose | L-arabinose | Ribose | D-xylose | Adonitol | B-methyl-xylitol | Galactose | D-glucose | D-fructose | D-mannose | L-sorbose | Rhamnose | Dulcitol | Inositol | Mannitol | Sorbitol | X methyl D | X-methyl D glucose | N-acetyl glucosamine | Arbutin | Esculin | Salicin | Cellobiose | Maltose | Lactose | Melibiose | Sacchrose | Trehalose | Inuline | Melezitose | D-raffinose | Amidone | Glycogene | Xylitol | B-gentiobiose | D-turanose | D-lyxose | D-tagatose | D-fucose | D-arabitol | D-arabiyol | Gluconate | 2-Cetogluconate | 5-Cetogluconate | Homo & Hetro | Growth at 15°C | Growth at 45°C | Growth at 4% NaCl | Probable Identity |
|----------|----------|------------|-------------|-------------|--------|----------|----------|------------------|-----------|-----------|------------|-----------|-----------|----------|----------|----------|----------|----------|------------|--------------------|----------------------|---------|---------|---------|------------|---------|---------|-----------|-----------|-----------|---------|------------|-------------|---------|-----------|---------|---------------|------------|----------|------------|----------|------------|------------|-----------|-----------------|-----------------|--------------|----------------|----------------|-------------------|-------------------|
| 1 | + | - | - | - | + | + | - | - | - | + | + | + | + | - | - | - | - | + | + | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | - | - | + | H | - | + | + | L | | |
| 2 | + | - | - | - | + | + | - | - | - | + | + | + | + | - | - | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | + | + | - | + | + | + | + | - | - | + | H | - | + | + | L |

Isolates: 1=*Lactobacillus plantarum*; 2=*Lactobacillus pentosus*. He = hetero fermenters =*Lactobacillus plantarum* and *Lactobacillus pentosus*; All the 3 strain were able to ferment ribose, glucose, fructose, N-acetyl glucosamine, Arbutin, esculin, salicine. The strain identified as *Lactobacillus pentosus* and *Lactobacillus plantarum* were able to ferment 28 sugar

The total Lactic Acid Bacteria (LAB) count of the 6 breast milk samples was determined. Result showed that sample D had the highest LAB count of 1.6×10^8 cfu/ml ($P > 0.05$) while sample A had the least LAB count of 1.3×10^6 cfu/ml ($P > 0.05$). The result is shown in Table 2.

After the antimicrobial activity on gastrointestinal pathogens which include; *Salmonella sp.*, *E. coli*, *Shigella sp.* and *Klebsiella sp.*, it was observed that all the intestinal bacteria were susceptible to the *Lactobacillus sps* (*L. plantarum* and *L. spentosus*) isolated from the breast milk. The result showed that *Klebsiella sp.* had the highest zone of inhibition of 16 ± 1.41 and 14 ± 8.48 (*L. plantarum* and *L. spentosus*), respectively; *Shigella sp.* had 14 mm and 10 mm (*L. splantarum* and *L. pentosus*), respectively and *Salmonella sp.* had 12 mm and 12 mm (*L. plantarum* and *L. pentosus*) while *E. coli* had the least zone of inhibition of 11 mm and 6 mm (*L. plantarum* and *L. pentosus*) in diameter. The zone of inhibition measured in diameter in millimeter

(mm) is shown in Figure 1.

DISCUSSION

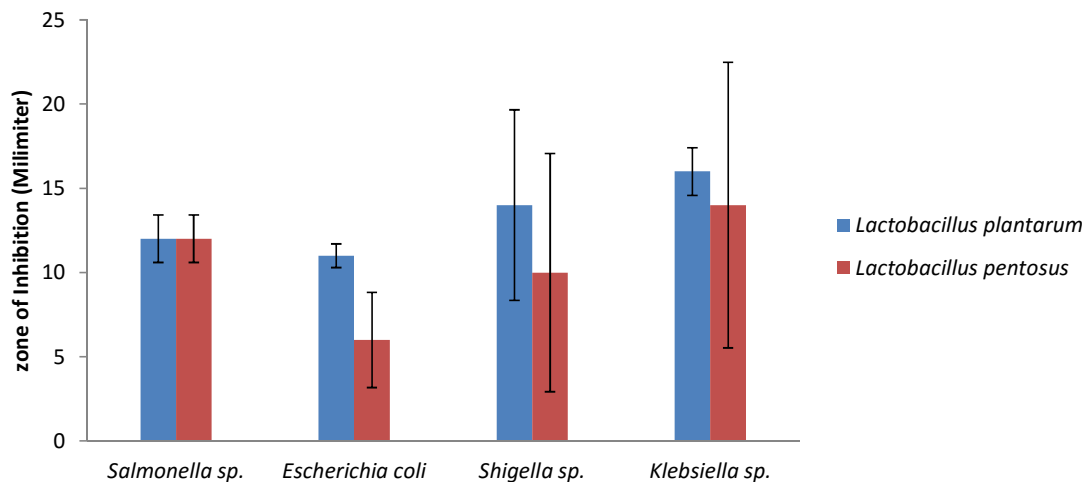
On the basis of colonial, morphological and biochemical characteristics (gram positive, catalase negative, endospore absence, non-motile, sugar fermentation pattern, bile tolerance activity and antimicrobial activity), using the API 50CHL, the isolates were identified as *L. plantarum* and *L. pentosus*. The colonies of *Lactobacillus* isolate are characterized to be *L. splantarum* and *L. spentosus* which appeared rough, dull white, 0.1-0.5 mm in diameter, and demonstrated medium to short rods. Earlier studies by Vamanu et al. (2005), Emanuel et al. (2005), Lilia et al. (2002), Oyetayo (2004), and Eduardo et al. (2003) have found similar aforementioned characteristics in isolated lactobacilli. Similar results have been also reported by Jara et al. (2011), and Heikkila and

Saris (2003).

The total Lactic Acid Bacteria (LAB) count of the 6 breast milk samples was determined. Result showed that sample D had the highest LAB count of 1.6×10^8 cfu/ml while sample A had the least LAB count of 1.3×10^6 cfu/ml ($P > 0.05$). The capacity of substances to inhibit microbial growth is referred to as antimicrobial activity. The result showed that *Klebsiella sp.* had the highest ($P < 0.05$) zone of inhibition of 16 ± 1.41 mm and 14 ± 8.48 mm (*L. splantarum* and *L. spentosus*), respectively; *Shigella sp.* had 14 ± 5.65 mm and 10 ± 7.07 mm (*L. plantarum* and *L. pentosus*), respectively and *Salmonella sp.* had 12 ± 1.41 mm and 12 ± 1.41 mm (*L. plantarum* and *L. pentosus*) while *E. coli* had the least ($P < 0.05$) zone of inhibition of 11 ± 0.7 mm and 6 ± 2.82 mm for *L. plantarum* and *L. pentosus* in diameter, respectively. This confirms the results obtained by Daniel et al. (2014) who isolated strains from dairy products, effective against this target organism. Inhibitory activity was demonstrated by some of all

Table 2. Total LABCount.

| Sample | Count | Dilution Factor | cfu/ml |
|--------|-------|------------------|-----------------------|
| A | 130 | 10 ⁻⁴ | 1.3 x 10 ⁶ |
| B | 150 | 10 ⁻⁵ | 1.5 x 10 ⁷ |
| C | 190 | 10 ⁻⁴ | 1.9 x 10 ⁶ |
| D | 160 | 10 ⁻⁶ | 1.6 x 10 ⁸ |
| E | 35 | 10 ⁻⁵ | 3.5 x 10 ⁶ |
| F | 190 | 10 ⁻⁴ | 1.9 x 10 ⁶ |

**Figure 1.** Antibiogram of lab isolates.

the test strains against *E. coli* 0157:H7, except for *S. thermophilus*. These findings are in agreement with that reported by Otopo and Ighanesebhor (1996) for inhibitory activities of human colostrum against *S. aureus* and coliform organisms. This may be due mainly to the high immunoglobulins (Igs) content of colostrum. Also, Azizpour et al. (1999) found that the breast milk exerted bactericidal activity was against *E. coli*, *P. aeruginosa* and *S. aureus*. *Salmonella sp.* inhibition halos were evident with some of all the lactic cultures. As the isolated lactic acid bacteria inhibited these pathogenic strains successfully, it may be expected that addition of these human milk probiotics to commercial food products for infants would confer effective protection against infections caused by these pathogens.

Conclusion

Lactic acid bacterial (*L. plantarum*) was isolated from human milk in pure culture and various properties of isolated bacteria were determined using the API50CHL identification kit. The Lab count was determined and the isolate showed antimicrobial activity against all the indicator microorganisms.

Recommendations

Future research work regarding adhesion to mucosal surface, clinical studies for human health, strain stability, bacteriophage resistance, and viability in products, antibiotic resistance should be carried out.

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