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IN VITRO STUDY ON THE ANTIMICROBIAL ACTIVITY OF PROBIOTIC MILK AGAINST COMMON PEDIATRIC COMMUNITY ACQUIRED RESPIRATORY PATHOGENS

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ABSTRACT

Objective: To determine the *in vitro* antimicrobial activity of probiotic containing milk against community acquired bacterial pathogens in the pediatric age group.

Design: Experimental Study

Methods: *In vitro* analysis of the antimicrobial activity of probiotic milk in comparison to antibiotics against control strains based on the Kirby Bauer Method of disc susceptibility testing. Kruskall Wallis Test was used to analyze the difference between the mean zones of inhibition of the different control groups. Chi square and Fisher Exact Test was used to analyze % susceptibility.

Results: Zones of inhibition of probiotic containing milk were observed among the three bacterial pathogens tested. The mean zones of inhibition produced by the probiotic milk showed smaller means for *Streptococcus pneumoniae* (9.04 +/-5.26) and *H. influenzae* (9.76 +/ - 7.65) as compared to their respective antibiotics: Penicillin G (30.18+/-0.70) and Chloramphenicol (30.96 +/- 1.86) (p value <0.000001). A larger mean zone of inhibition was produced for *Staphyloccocus aureus* (16.96+/- 5.30) compared to Oxacillin (12.92+/- 0.65). Comparison of the % susceptibility showed higher susceptibility of *Streptococcus pneumoniae* to Penicillin G and *H. influenzae* to Chloramphenicol when compared to the probiotic containing milk. *Staphyloccocus aureus* however, showed a better susceptibility to the probiotic containing milk (88%) vs. Oxacillin (64%) with a P value of 0.04.

Conclusion: Probiotic milk containing *Lactobacillus* and *Bifidobacteria* was observed to have in vitro antimicrobial activity against *Streptococcus pneumoniae, H. influenzae,* and *Staphyloccocus aureus* . It has a better antimicrobial activity against *S. aureus* as demonstrated by a larger zone of inhibition and increased proportion of disc susceptibility than Oxacillin.

INTRODUCTION

There has been an increasing scientific and commercial interest in the use of beneficial microorganisms, or probiotics for the prevention and treatment of disease. For many years, probiotics such as *Lactobacillus* and *Bifidobacteria* in food products have been touted for their reputed health benefits. However, scarcity of supporting evidence for these health effects was, in previous years, largely anecdotal. Until recently, evidence has started to accumulate, as studied in literature, from good quality clinical control trials with randomized, placebo controlled design; and deductions from well-founded in vitro studies.

The use of probiotics to control certain infections has, likewise, started to gain acceptance. The alarming rise of inappropriate antibiotic use, and antimicrobial resistance, along with renewed interest in ecological methods to prevent infections, makes probiotics a very interesting field for research. Latest studies to date on their potential use in infectious disease include an *in vitro* study showing comparable antimicrobial activity of probiotic containing milk with that of breastmilk against common bacterial isolates in a hospital setting.[1] Another double blind, randomized controlled trial in daycare centers studied the effect of probiotic milk on diarrhea and respiratory infections in children, and reported for the first time reductions in respiratory infections and their severity.[2] The extent to which probiotic containing milk is able to exert activity against common bacterial pathogens, however, has not been studied. Because of this new benefit, an *in vitro* study to investigate the antimicrobial activity of probiotic- containing milk against common community acquired bacterial pathogens in children is being undertaken. A comparison of the antimicrobial activity of the commonly used antibiotics against these pathogens *in vitro* also needs to be determined.

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OBJECTIVES

General Objectives

This study was undertaken to determine the *in vitro* antimicrobial activity of probiotic- containing milk against community acquired bacterial pathogens in the pediatric age group.

Specific Objectives

1. To determine the *in vitro* antimicrobial activity of probiotic-containing milk against pediatric community acquired bacterial pathogens, namely: *H. influenza, S. pneumoniae,* and *S. aureus*
2. Compare the *in vitro* antimicrobial activity of probiotic-containing milk versus Chloramphenicol, Penicillin G, and Oxacillin based on zones of inhibition and on known susceptibility patterns of *H. influenza, S. pneumoniae,* and *S. aureus* to these antimicrobials

MATERIALS AND METHODS

Samples

One commercial milk formula containing probiotics *Lactobacillus* and *Bifidobacteria* (Gain Plus, ABBOT Pharmaceuticals) was prepared as instructed by its manufacturer using sterile water and containers. Using sterile syringes, two ml of prepared milk formula was transferred into sterile plastic petridishes ready for use.

The pathogens used as reference bacterial strains are control srains as follows: *Haemophilus influenzae* ATCC 49247, *Steptococcus pneumoniae* ATCC 41619, and *Staphylococcus aureus* ATCC 25922 obtained from the Microbiology and Infectious Disease Center in Alabang

Controls

Sterile water was used as negative control. The antibiotics Chloramphenicol, Penicillin G and Oxacillin were used as positive control.

Preparation of the Culture Media

Standardization of the culture media and preparation of the control strains through broth cultures was done by a single senior medical technologist in accordance with the National Committee for Clinical Laboratory Standards (NCCLS 2000). Probiotic-containing milk and Antibiotic Susceptibility Testing

For testing susceptibility of the bacterial pathogens, the Kirby Bauer Disk Diffusion method was employed. 75 sterile blank 6 mm disks were dipped into the probiotic -containing milk preparations then placed and equally distributed on the prepared MHA culture media streaked with the three respiratory pathogens alongside antibiotic disks specific for a particular bacteria; Penicillin G for *S. pneumoniae*, Chloramphenicol for *H. influenza* , and Oxacillin for *S. aureus* . The same was done for sterile water which was used as negative control. All disks were incubated for 18-24 hours at a temperature of 37 ^{0}C. Measurement of the zones of inhibition produced by the probiotic-containing milk, antibiotics (positive control), and sterile water (negative control) were done on the 24th hour using a standard caliper of 0.05 mm. Results were recorded and tabulated accordingly.

Outcome Measurement and Data Analysis

In vitro antimicrobial activity of probiotic containing milk against common community acquired bacterial pathogens was measured by zones of inhibition

1. Measurement of the mean zones of inhibition of milk containing *Lactobacilli* and *Bifidobacteria*
2. Measurement of the mean zones of inhibition of the negative and positive controls
3. Comparison of the mean zones of inhibition of probiotic-containing milk and known zones of inhibition based on standard susceptibility patterns of antibiotics: Chloramphenicol, Pen G, and Oxacillin against *H. influenza, S. pneumoniae,* and *S. aureus,* respectively, was done using the Kruskal Wallis Test and Mann Whitney U test. A p value of <0.05 was considered statistically significant.
4. Measurement of the percentage susceptibility defined as proportion of disks showing susceptibility of test organisms to antibiotics an probiotic containing milk, based on standard susceptibility patterns of the antibiotics use for each organism was compared using Chi-square test and Fisher Exact Test

RESULTS

This study included one commercial milk formula containing the probiotics *Lactobacillus salivarius* and *Bifidobacteria;* and control strains namely, *S. pneumoniae, H. influenzae* and *S. aureus*

The zones of inhibition produced by the probiotic-containing milk , Penicillin G, Chloramphenicol, and Oxacillin against the these pathogens were compared. The zone of inhibition produced by the milk is a measure of its activity to the reference bacteria, with a larger value interpreted as representing a better in vitro antimicrobial activity.

Table 1 and 2 showed a significant difference in the mean zones of inhibition between the probiotic-containing milk and Penicillin G (p value <0.000001).

Using established standard susceptibility patterns, zones of inhibition produced by the milk and Penicillin G for *Streptococcus* were labeled as susceptible if it had a zone of ≥ 20mm, 29 mm for Chloramphenicol against H. influenza, and 13 mm for Oxacillin against *Staphylococcus aureus.*

Table 1. Comparison of the Mean Zone of Inhibition for *Streptococcus pneumoniae*

	Probiotic-containing Milk	Sterile Water	Penicillin	P Value
Mean +/- SD Median	9.04 ± 5.26 6	6.0 ± 0 6	30.18± 0.70 30	<0.000001 (S) Kruskall Wallis Test

Table 2. P Values for the Comparison of Two Groups

Variables	*p* value
Probiotic- containing Milk Vs Sterile Water	0.005
Probiotic- containing Milk Vs Penicillin G	0.000001
Penicillin G Vs Sterile Water	0.000001

For *Hemophilus influenzae,* the result likewise, showed a significant difference in the mean zones of inhibition. When compared to Chloramphenicol, the probiotic-containing milk was noted to have a weak *in vitro* antimicrobial activity against this pathogen. (Tables 3 & 4)

Table 3. Comparison of the Mean Zone of Inhibition for Hemophilus influenzae

	Probiotic-milk	Sterile water	Chloram-phenicol	P value
Mean +/- SD Median	9.76 ± 7.65 6	6.0 ± 0 6	30.96± 1.86 30.5	<0.000001 (S) Kruskall Wallis Test

Table 4. P Values for the Comparison of Two Groups

Variables	P Value
Probiotic- containing Milk Vs Sterile Water	0.009
Probiotic- containing Milk Vs Chloramphenicol	0.000001
Chloramphenicol Vs Sterile Water	0.000001

Mann Whitney U Test

For *Staphylococcus aureus,* comparison was significantly different. Compared to Oxacillin, the mean zone of inhibition produced by the probiotic containing milk was significantly larger, hence, better antimicrobial activity. (Table 5 & 6)

Table 5. Comparison of the Mean Zone of Inhibition for Staphylococcus aureus

	Probiotic-containing Milk	Sterile Water	Oxacillin	P Value
Mean +/- SD Median	16.96 ± 5.30 15	6.0 ± 0 6	12.92± 0.65 30	<0.000001 (S) Kruskall Wallis Test

Table 6. P Values for the Comparison of Two Groups

Variables	P Value
Probiotic- containing Milk Vs Sterile Water	0.00004
Probiotic- containing Milk Vs Oxacillin	0.0004
Oxacillin Vs Sterile Water	0.000001

When the proportion of the % susceptibility was compared between the 3 groups using Chi- square , significant differences were noted. *Streptococcus pneumoniae* showed a 100% susceptibility to Penicillin G, with only 8% to the probiotic containing milk. *H. influenzae* showed an 87.5% susceptibility to Chloramphenicol as against 8% with the milk tested. Staphylococcus aureus, on the other hand, showed a

better susceptibility pattern to the probiotic containing milk compared to Oxacillin: 88% vs. 64%. (Table 7)

Comparison of the % susceptibility between two groups using the Fisher Exact Test showed similar results. Moreover, the results further show that the % susceptibility of *S. aureus* to probiotic containing milk is significantly higher than Oxacillin. (p value 0.04).

Table 7. Comparison of the % Susceptibility

	Probiotic Containing Milk	Sterile Water	Anbtibiotic	*p* value
Streptococcus pnueumoniae	2 (8.0%)	0 (0%)	25 (100%) (Penicillin G)	0.000001 (Significant)
H. influenzae	2 (8.0%)	0 (0%)	7 (87.5%) (n=8) (Chloram-phenicol)	0.000001 (Significant)
Staphylococcus Aureus	22 (88.0%)	0 (0%)	16 (64.0%) (Oxacillin)	0.000001 (Significant)

Table 8. P Values for the Comparison of the % Susceptibility Between Two Groups

	Streptococcus Pneumoniae	*Hemophilus influenzae*	*Staphylococcus aureus*
Probiotic-containing Milk Vs Sterile Water	0.49 (Not Significant)	0.49 (Not Significant)	0.000001 (Not Significant)
Probiotic-containing Milk Vs Antibiotic	0.000001 (Significant)	0.00006 (Significant)	0.04 (Significant)
Antibiotic Vs Sterile Water	0.000001 (Significant)	0.000001 (Significant)	0.000001 (Significant)

DISCUSSION

Increased risk of disease in the pediatric age group has obvious public health and economic consequences, such as direct medical costs as well as indirect costs of parents having to take time off from work to look after sick children.[3] The successful prevention of infections could be extremely useful for families and for society in general. Since prevention would obviate the need for treatment, the use of probiotic bacteria to prevent common childhood infections have been proposed.[4]

In ancient times, the benefit and health potential of foods containing live bacteria have been recognized. During the beginning of the 20th Century, Elie Metchnikoff proposed a scientific rationale for the beneficial effects of bacteria in yogurt and attributed long life to intake of yogurt containing Lactobacillus species. Since then, multiple antimicrobial properties have been suggested.

The word 'probiotic' is derived fron the Greek, 'for life'.[5] Today, they are defined as live microbial food supplements with a demonstrated effect on human health. There are several commercially available supplements contaning probiotics, either as fermented food products or dairy based foods. Recently, with the advancement in research and infant nutrition, it has been introduced in some milk formulas and powdered milk supplements.

The main mechanisms whereby probiotics exert protective or therapeutic effects are not fully elucidated, but multiple mechanisms have been postulated including lactose digestion,[6] production of antimicrobial agents,[7,8] competition for space nutrients, and immunomodulation: adjuvant like effects on intestinal and systemic immunity[9] and non-humoral immunity.[10]

In a review of recent studies, probiotic therapy showed substantial evidence of clinical benefit in pediatric patients with viral gastroenteritis and prevention of nosocomial diarrhea;[11] in the treatment of *Clostridium defficile* diarrhea.[12] The use of Lactobacillus GG was also found to be effective in the prevention of early atopic disease in children with high risk.[13] These clinical studies have attempted to establish the value of probiotics in the prophylaxis and treatment of childhood diseases. Fewer studies have been done to evaluate their role in the prevention of community acquired infections. In one such study, long term consumption of probiotic milk was found to be beneficial in reducing incidence of diarrhea and respiratory infections.

To evaluate further the probiotics' potential in infections, the in vitro antimicrobial activity of a probiotic milk containing *Lactobacillus and Bifidobacteria* against common bacterial respiratory pathogen was determined. Zones of inhibition were observed in all the three organisms tested. However, among these 3 organisms, only Staphylococcus aureus was found to be susceptible to the probiotic containing milk with a more superior antimicrobial activity over Oxacillin. In a similar study comparing the same probiotic containing milk and breastmilk, zones of inhibition were likewise larger for *S. aureus* , and the observed antimicrobial activity was comparable to breastmilk.

The significant antimicrobial activity of the probiotic containing milk observed in this experimental study may shed light on an interesting case report in 1997 which describes successful treatment of a decubitus edcer colonized by MRSA with the use of a Lactobacillus preparation.[14] Studies of this potential use may have profound impact in the coming years.

CONCLUSION

In conclusion, milk containing *Lactobacillus* and *Bifidobacteria* has *in vitro* antimicrobial activity against *S. pneumonia* and *H. influenzae* but less than Penicillin amd Chloramphenicol, respectiveley. It has better antimicrobial activity against *S. aureus* as demonstrated by larger zones of inhibition and increased proportion of disc susceptibility to than Oxacillin.

RECOMMENDATION

This experimental study has presented data showing that probiotic containing milk has a significant antimicrobial activity against *Staphylococcus aureus*. Further research is required to delineate this potential. It is therefore recommended that clinical in vivo studies be done to measure this in vitro advantage, in order to translate it to clinically relevant outcomes.

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