Development of Biotherapeutic Agent for the Treatment and Prevention of Gastroenteritis in Children

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Key words: **Acute diarrhea, Bifidobacteria, probiotic, Infant, Gastroenteritis**

Introduction

Infectious diarrhea is a worldwide public health problem. In many developing countries, diarrheal diseases remained a leading cause of illness and death among infants and children. Bacterial pathogens probably cause less than 20 % of cases of acute childhood diarrhea. Globally, *Salmonella, Shigella* and *Campylobacter* remain major contributors to diarrheal diseases. Some strains of *Escherichia coli*, a normal inhabitant of the distal bowel, are pathogenic, causing sporadic cases of acute enteritis, epidemic diarrhea (particularly in young infants) and traveler's diarrhea. Severe diarrhea in children has now been attributed to enteroadherent strains of *E. coli*. In Malaysia, investigation for common diarrheal bacteria has not much been documented.

Numerous probiotic agents have been studied in the management of infectious diarrheal. Preliminary experimental and clinical findings show that probiotics are emerging as an important, new therapy for preventing and treating infectious diarrhea. Ingestion of probiotics can exert a positive influence on the health or physiology of the host. It was believed that it could influence intestinal physiology either directly or indirectly through modulation of the endogenous ecosystem or immune system.

Bifidobacteria, a probiotic, comprised a major group in the human and animal intestinal flora along with bacteroides and eubacteria. They are thought to exert some of the protective effect against acute diarrhea diseases. Bifidobacteria are Gram positive, non-acid fast, non-spore forming and non-motile organism. These organisms have been isolated from the faeces of breast-fed infant, adult human intestine, vagina and mouth as well as in the alimentary tract of various kinds of animal. In the large intestine, bifidobacteria produce acetic and lactic acids and is thought to inhibit the proliferation of putrefactive bacteria such as escherichiae, clostridia and eubacteria.

In order to use bifidobacteria as an alternative to manage acute diarrhea, it is necessary to establish the strain that can survive in the acidic conditions of the stomach. And also, the dose of the bifidobacteria that is able to reduce the growth of the causative microorganism should also be established. Considering these reasons, the objectives of the present study are:

To isolate and characterize the major diarrheagenic pathogens from stool of acute diarrhea patients below 3 years of age

To study the dose-effect of *Bifidobacterium* spp. against the *Salmonella* spp. in simulated human colon environment

Materials and Methods

Thirty stool samples were collected from children, aged 3 years or younger, admitted to K7 ward, Pediatric Institute, Kuala Lumpur Hospital. Stools samples were collected using sterile swabs and were placed in 5 ml Cary-Blair (Oxoid-Unipath Ltd., Basingtocke, Hampshire England) transport medium, and sent on wet ice to Microbiology Laboratory, Faculty of Food Science and Biotechnology, Universiti Putra Malaysia. The specimens were surface plated as soon as possible, generally within 24 hrs, onto eight different types of selective agar. Gram staining and morphology observation was performed according to the standard method of Medical Laboratory Manual by Cheesbrough (1984). The carbohydrate fermentation pattern was studied using a commercial API 20E Kit (BioMeriux, France) for identification of *Salmonella* spp., *E. coli*, and *Yersinia enterocolitica*.. Antimicrobial susceptibility tests for *Salmonella* were performed using Stokes disc diffusion technique (Stokes and Ridgway, 1980) on Mueller-Hinton agar according to NCCLS recommendations (1993). Sixteen pure isolates of purified *Salmonella* were sent to Institute for Medical Research (IMR) at Jalan Pahang, Kuala Lumpur, Malaysia for serological test. The test was done by agglutination technique. PCR-based fingerprinting was carried out in volumes of 25 µl containing 20-30 ng of *Salmonella* spp. total DNA, 2.5 mM $MgCl_2$, 5 pmol of primer, 2.5 U of Taq DNA polymerase (Promega, USA), and 250 mM each of dATP, dCTP, dGTP and dTTP (Promega, USA). Two probiotic bacteria used were *B. infantis* ATCC 27920 and *B. pseudocatanulatum* F117 and the two diarrheal bacteria used were *S. enterica* ser. Enteritidis S260 and *S. enterica* ser. Hindmarsh 4F1. The experiments were performed as therapeutic relationship between the probiotic cultures at three different concentrations and the diarrheagenic pathogens at maximum level. The quantitative analysis of organic acids (acetic and lactic acid) in the sample broth was analyzed using the High Performance Liquid Chromatography (HPLC) method. The HPLC system includes a Shimadzu LC10AS Liquid Chromatograph (Shimadzu, Japan), 6000A pump, U6K injector, an organic acid analysis column (HP-87H: Bio-Rads Laboratories, Richmond, USA) and a chart recorder (Shimadzu, Japan). The oven temperature was set at 40 °C. The eluent used was 0.008 N Sulphuric Acid at a flow rate of 0.7 ml/min.

Results and Discussion

A total of 152 faecal bacterial isolates suspected as diarrhoeagenic pathogens were recovered from this study. From biochemical tests, gram staining and morphological studies, 120 isolates were identified as *E. coli* and they were recovered from all the 30 subjects. 15 isolates of *Salmonella* spp were identified and obtained from 4 subjects, whilst 8 isolates identified as *Yersinia enterocolitica* and *Proteus* spp were recovered from 2 subjects. As *E. coli* is among the most common bacteria in the normal flora of the human intestine besides the other pathogenic strains and consequently *Salmonella* spp. was the highest exogenous species isolated and therefore, have been chosen for further study. All the 15 isolates of *Salmonella* spp. were grouped into 3 serovars. They were 5 isolates of *S. enteritica* serovar Akanji isolated from 1subject, 7 isolates of *S. enteritica* serovar Hindmarsh isolated from 2 subjects and 3 isolates of *S. enteritica* serovar Richmond isolated from 1 subject. The antibiotic susceptibility test showed that all the *Salmonella* spp. isolates were highly susceptible to amoxicillin, ampicillin, carbenicillin, cefuroxime, ceftaxidime, cephalotin, chloramphenicol, ciprofloxacin, nalidixic acid, and tetracycline, but mildly susceptible to gentamycin, kanamycin, streptomycin, and penicillin. All the isolates were resistant to bacitracin and erythromycin. Both of the control strains were highly susceptible to ceftaxidime, mildly susceptible to cephalotin, cefuroxime, and gentamycin and resistant to all others antibiotic excluding kanamycin and nalidixic acid. *S. enteritidis* S260 was resistant to kanamycin but susceptible to nalidixic acid, while *S. typhimurium* S974 was mildly susceptible to kanamycin but resistant to nalidixic acid. Ten oligonucleotide decamers were tested for their ability to generate RAPD markers from genomic DNAs of a subset of 4 *Salmonella* strains. Seven primers gave either non-reproducible patterns or gave very poor amplification. Primer P3, P9 and P10 yielded clear and reproducible patterns and were chosen for further analysis. To obtain a comprehensive result of the intraspecific relationships between all the strains analyzed, the data from RAPD-PCR patterns obtained with the three primers were combined in a single dendrogram. Two main clusters were observed. Cluster 1 comprised 12 strains and cluster 2 contained 3 strains.

In non-control pH experiment, growth of *Salmonella* spp. was inhibited by proliferation of *Bifidobacterium* spp. in all the combinations and dosages. The minimum viable count of both *S. enterica* ser. Hindmarsh 4F1 and *S. enterica* ser. Enteritis S260 after cultivated with *B. pseudocatanulatum* F11 were 10^7 and 10^8, respectively for all dosages. When *B. infantis* ATCC 27920 was used as bio-therapeutic agent, the minimum viable count of both *Salmonella* strains were 10^8 at dose 1 and dose 2 but it was 10^7 at dose 3. However, at different dosages of bifidobacteria, the minimum viable count has been reached at different time interval.

This study as well, indicates that higher concentration of bifidobacteria need longer time to inhibit the salmonella to the minimum level. Moreover, the duration on achieving of maximum inhibition was depends on tested and target strains. It seems like *B. pseudocatanulatum* F117 more effective to inhibit *S. enterica* ser. Enteritis S260 while *B. infantis* ATCC 27920 more effective to inhibit *S. enterica* ser. Hindmarsh 4F1 in term of time required. From the observation, the inhibition activities were absolutely effect by decreasing of pH values. Generally, at certain time interval the pH would be increased back and the viable count of bifidobacteria would be decreased.

Changes of pH values were noticed in the entire combinations and dosages. The pH values were decreased with increasing of time. The minimum pH associated with all dosages of *B. pseudocatanulatum* F117 were depends on resident strain but not for *B. infantis* ATCC 27920. In cultivation with *S. enterica* ser. *Akanji* 3C1 as a resident strain, the minimum pH that has been achieved was 4.0, while if *S. enterica* ser. Enteritidis S260 as a resident strain, the minimum pH was 4.2. In contrast, the minimum pH associated with all dosages of *B. infantis* ATCC 27920 was 4.6 when inoculated to both resident strains.

When the pH of the cultivation medium was control all through the experiment at pH 5.5 - 6.0, there were decreased in viable count of *Salmonella* spp. as well. The trend of decreasing was almost the same as the non-control pH but they were 1 log and 2 logs higher when cultured with *B. pseudocatanulatum* F117 and *B. infantis* ATCC 27920 respectively. Moreover, the times they take to inhibit the *Salmonella* spp. to the minimum viable count were longer. This was likely disagreed with the fact that the decreasing on pH values of the medium solely attributed inhibition activity of bifidobacteria. Furthermore, there was no declining of bifidobacteria viable counts at the end of the experiments were observed.

In non-control pH batch, production of acetate and lactate by the *Bifidobacterium* strains was observed. Roughly, it looks like the production of those organic acids was higher when the initial count of bifidobacteria was lower . *B. pseudocatanulatum* F117 was produced more acetate and lactate when cultivated with *S. enterica* ser. Enteritidis compared to S260 *S. enterica* ser. Hindmarsh 4F1. While *B. infantis* ATCC 27920 was produced more lactate but less acetate when cultivated with *S. enterica* ser. Hindmarsh 4F1 compared to *S. enterica* ser. Enteritidis S260. In overall, *B. pseudocatanulatum* F117 was produced more organic acid compared to *B. infantis* ATCC 27920.

The duration to produced the maximum concentration of acetate was not depends on the dosage of *Bifidobacterium* species. All the combination and dosages were produced the maximum concentration of acetate in 48 hrs except *B. infantis* ATCC 27920 versus *S. enterica* ser. Enteritidis, which the duration was 54 hrs. However, lactate production was depends on the dosage of bifidobacteria and the target strain. The results demonstrated that dose 2 have the minimum duration time in producing the maximum concentration of lactate. Dose 1 and 3 has the same duration time except *B. infantis* ATCC 27920 versus *S. enterica* ser. Hindmarsh 4F1 where dose 1 have longer duration time compared to dose 3. The concentrations of organic acids were generally decreased at the end point of the experiment.

Conclusions

This study was designed to investigate the inhibitory activity and dose effects of selected probiotic microorganisms against common diarrheal bacteria associated with acute diarrhea in children under 3 years old, who where admitted to Pediatric Institute, Kuala Lumpur Hospital. The first part of the study involved the isolation and identification of diarrheal bacteria from

stool sample of infected children. The results suggest that *Salmonella* spp. was one of the etiologies as they were the main pathogen isolated from children with acute diarrhea at Pediatric Institute, Kuala Lumpur Hospital.
In the second part of the study, the survival of bifidobacteria in the human stomach at the pH after meal compared to the pH before meal (fasted state) was evaluated. The results demonstrated that those probiotic could tolerate in both stomach acidity, however the tolerance was better in the pH range after meal consumption. The survival counts of both conditions have been used as the dosage of these probiotic in dose effects study.

Benefits from the study

An alternative technique in the prevention and treatment of gastro-enteritis. The technique exploits GIT microbial relationship. And develop a technique to reduce the incidence of acute diarrhea and rotavirus shedding in infants and children. So, infant and children diarrhea, which has been a major cause of mortality can be reduced

Patent(s), if applicable:

Nil

Stage of Commercialization, if applicable :
Nil

Project Publications in Refereed Journals

1. **Anis Shobirin M. H.**, Shuhaimi M., Abu-Bakar F., Ali A. M., Ariff A., Nur-Atiqah N.A. and Yazid A.M. 2003. Characterization of *Salmonella* spp. isolated from patients below 3 years old with acute diarrhoea. World Journal of Microbiology and Biotechnology. 9(7): 751-755.

2. Anis Shobirin M. H., Shuhaimi M., Ali A. M., Ariff A., and Yazid A.M. 2003. Inhibitory Activity of *Bifidobacterium pseudocatanulatum* against *Salmonella* spp. Journal of Food Protection. In Progress

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Project Publications in Conference Proceedings

Anis Shobirin, M.H., Shuhaimi M., **Yazid, A.M.**, Ali, A.M. and Ariff, A. 2002. Acute diarrhea in children under 3 years old admitted to the Pediatric Institute, Kuala Lumpur Hospital. Proc. *Paper presented at 25th Malaysian Micribiology Society Symposium and 5th UNESCO national Workshop on the Promotion of Microbiology in Malaysia*. 8-11 September 2002, Kota Bharu, Kelantan, Malaysia. pp 18.

Anis Shobirin M. H[1]., A.M. Yazid[1], N.A. Nur Atiqah[2], A. M. Ali[1] and A. Ariff[1]. 2002. Perencatan Salmonella oleh Bifidus. Ulang Tahun ke-25 Fakulti Sains Makanan dan Bioteknologi, Universiti Putra Malaysia. 12-14 Julai 2002. Serdang, Selangor, Malaysia.

Graduate Research

Name of Graduate	Research Topic	Field of Expertise	Degree Awarded *(e.g. M.SC/Ph.D.)*	Graduation Year *(or expected)*
Anis Shobirin Meor Hussin	Inhibitory Activities of a Probiotic Bacterium (*Bifidobacterium pseudocatanulatum*) on a Common Diarrheagenic Pathogen (*Salmonella enterica*) in Human	**Food Microbiology**	**M.SC**	**2003**

Shuhaimi Mustafa	Species Classification and Molecular Studies of Bile Salt Hydrolase (bsh) Gene in Bifidobacterium spp	**Food Microbiology**	**Ph.D**	**2002**

IRPA Project number03-02-04-0118
UPM Research Cluster:AFF
Project Leader Assoc. Prof. Dr. Mohd Yazid ABD Manap