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Research Article

# EFFCT OF SYNBIOTIC MILK BEVERAGE ON DIARRHOEAL CAUSING ORGANISMS

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**ABSTRACT**

The aim of this research was to evaluated that effect of synbiotic milk containing oligosaccharide to enhance the growth and activity of probiotic strains include *Lactobacillus kefiranofaciens, Candida kefir* and *Saccharomyces boluradii* were testd for their antibiotic susceptibility, tolerance to bile, and antidiarrhoeal effect. Antimicrobial activity of synbiotic milk could differ in their antagonistic activities against diarrhoeal causing orgnisms which could be due to the metabolite secreted by the lactic acid bacteriocin specially type of organic acids and added inulin as a prebiotic and for food preservation. Among the five milk beverage the best combinational approachment of milk beverage was identified and their major compounds was detected using Gas chromatography – Mass Spectrum (GC-MS).

**Keywords:** Synbiotics, Inulin, Food preservation, Lactic acid bacteria, GC-MS analysis.

## INTRODUCTION

Diarrhoea is a common symptom of intestinal disorders and it is a global threat to human health. It is a leading cause of morbidity and mortality, with over 1000 million episodes and over 4 million deaths annually in children under 5 years of age. Diarrhoeal infection is a second killer disease of children in the developing countries. Diarrhoea caused by *Escherichia coli* is common in India with occasional outbreaks Kahali *et al.,* (2004). Whereas *Escherichia coli* (58.4%) *Salmonella* sp (20%) and *Shigella* sp (20%) were found to be extremely uncommon agents of childhood diarrhea making only 1.6 per cent of the positive culture in Yeman. Banajeh *et al.,* (2001). A synbiotic is a supplement that contains both a prebiotic and probiotic that work together to improve the friendly flora of the human intestine. Reasearch and development of synbiotic products have been increasingly focusing on evidence of functional benefits including resistance to infection, antibacterial activity, and improved immune status( Gibson and Roberfroid, 1995). Probiotics are live microbial food supplements which beneficially affect the host by improving the intestinal microbial balance Tannock *et al.,* (2000).

As such, probiotic bacteria should have the ability to resist the digestion process in the stomach and the intestinal track. After the pass through the stomach, they enter the upper intestinal track where bile is secreted into the gut. The concentration of bile in the human gastrointestinal system is variable and is difficult to predict at any given movement Lankaputhra and Shah (1995). After traveling through this harsh environment the organisms colonize the epithelium of the lower intestinal track that should be able to tolerate acid and bile acids, attach to epithelium, providing health benefits.

Probiotics are nonpathogenic, technologically suitable for industrial process, and produce antimicrobial substances including organic acids, hydrogen peroxide and bacteriocins (biologically active protein) Dunne *et al.,* (1999). The objective of the study was to isolate and identify the beneficial bacteria (probiotics) from fermented milk samples such as yoghurt, kefir, butter, cheese and koumiss. Five species of probiotics isolated, and its effective combinational approachment is identified and inoculated with five different milk samples of Cowmilk, Buffalomilk, Lactogen, Hatsun, NAN (Skim milk powder) and after fermentation to treat against diarrhoeal causing organisms. Because milk have milk solids, minerals, soya lecithin and vitamins and proteins responsible for growth for bones and teeth, blood formation, mental development, immune system development and eyesight development of infants. Lactic acid bacteria make up extremely important group of probiotic bacteria and are already used in many probiotic dairy products Lourens–Hattingh and Viljoen (2001).

To evaluate prebiotics strains for their compatibility with milk sample in the presence of inulin might be a good source of probiotics and also nutritional components even after 4 weeks storage at 4°C.In addition it would be completely functional when ingested. After 4 weeks storage at 4°C the amount of microbes present was still sufficient to give probiotic charcteristics to the milk**.** Among prebiotics, non-digestable carbohydrate like inulin and oligofructose has received much attention. Inulin consists of 2-60 fructose units linked by a β-(2-1) glycosidic linkage often with a terminal glucose unit. Many researches proved that consumption of prebiotics, such as inulin, could stimulate intestinal peristalsis by means of increasing fecal bulk and moisture Gibson *et al.,* (1995). The keeping above facts in view present investigation was undertaken to evaluate prebiotics strains for their compatibility with milk sample in the presence of inulin for synbiotic milk beverage preparation.

## MATERIALS AND METHODS

### Sample Enrichment

Fermented milk sample was collected from market and was used for isolating probiotic bacteria and yeast. The milk sample was inoculated and allowed to ferment at room temperature for a week spontaneously without any additives through the milk endogenous micro organisms. The enrichment process of the collected organisms inoculated same was carried out as follows, low volume of inoculated milk was added to 80ml MRS broth (Man rogosa sharpe) in 150ml conical flask. The enriched sample was incubated under anaerobic conditions. The enrichment process was conducted in triplicate and repeated on weekly basis for one month period.

### Probiotic Isolation and Characterisation

The isolation process was carried out by streaking the enriched samples on MRS agar media and incubated at 37°C Ayad *et al.,* (2004). The five probiotics were characterized and identified using colonly morphology, biochemical test and in selective medium, carbohydrate fermentation (Table 1). From five which three probiotic species were namely *Lactobacillus kefiranofaciens, Candida kefir* and *Saccharomyces boluradii* were identified to effective against diarrhoeal causing pathogens, The best combinational approachment of probiotic bacteria and yeast confirmed by using acid tolerance test, bile tolerance test and cell adhesion test, Antimicrobial activity test etc.,

### Probiotication of Synbiotic Milk beverage

Milk samples of cow, buffalo, Lactogen, Hatsun, NAN were taken in sterile container. Milk samples were pasteurized and homogenized properly and 100ml of milk samples were inoculated with 2ml of MRS broth containing probiotic bacteria and yeast. (i.e *Lactobacillus kefiranofaciens, Candida kefir* and *Saccharomyces boluradii)* they

were allowed for fermentation. After fermentation, milks were separated into two different containers. One of that container inulin could be added and tested for antibacterial activity.

**Bile Tolerance**

Probiotic strains were cultivated in MRS broth enriched with 2% of oxgall (dehydrated bile, Becton and Dickinson) at 37˚C for 24h. The growth was checked by spreading of 100µl of cultures of appropriate dilution on to MRS agar (oxoid). Control cultures were without oxgall and cells counts were compared with those after 24h. Bacterial growth was expressed in colony forming units per milli liter (cfu/ml).

**Tolerance to Acetic pH values**

Probiotic strains were grown in MRS broth (oxoid) at 30˚C overnight, then subcultured into fresh MRS broth and incubated for another 24h. The cultures were centrifuged at 5000g for 10mins at 4˚C. The pellets washed in sterile phosphate buffered saline (PBS) pH 7, and resuspended in PBS. Each probiotic strain was diluted 1/100 in PBS at pH 1, 2 and 3. Incubation times were 2, 4 and 6h. Bacteria were then transferred to MRS broth (oxoid) and incubated at 37˚C overnight El-Naggar (2004). Counts of surviving cells were determined by plating on MRS agar(oxoid) bacterial growth was expressed in colony forming units per milli liter (cfu/ml). the experiments was repeated twice and each reading represents the mean of three observations.

**Detection of Antagonistic activity of Probiotics**

**Test Organisms**

The bacteria used as test organisms were *Staphylococcus aureus, Escherichia coli, Vibrio cholerae, Salmonella paratyphi A*, and *Shigella dysenteriae*. These were purcured from MTCC (Microbial Type Culture Collection) IMTECH, Chandigarh, India.

**Preparation of Inoculum**

Inoculum was prepared by adding one loopful of test pathogen in 50ml of BHI ( Brain Heart Infusion) broth and then incubated at 37˚C for 24hrs

**Agar Well Diffusion Method**

The antibacterial activity of synbiotic milk beverage was evaluated by agar well diffusion method Chung *et al.,* (1990). Muller Hinton agar medium was prepared and poured into the petriplates and allowed to solidify. Then it was inoculated with a swab of culture and spread throughout the medium uniformly with a sterile cotton swab. Using sterile cork borer (10mm diameter) wells were made in the agar medium. The test compound was introduced into the separate well in a single plate.( Non fermented milk, fermented milk, fermented milk with inulin) All the plates were incubated at 37˚C for 24h. The antagonistic test was performed intriplicate and their efficiency was determined by measuring the diameter of zone of inhibition around the well. In triplicate assay mean value was taken for analysis.

**GC-MS analysis**

The volatile constituents from milk beverage was analysed using GC-MS (GC Clarus 500 Perkin Elmer) with Elite-1 column and a mass detector, which was operated in EI mode at 70eV. Injector and detector temperatures were set at 250˚C Al-Delaimy and Ali, (1970). Plums juice (1µl) was injected and analysed with a column held initially at 110˚C for 2min and then increased by 5˚C per min up to 280˚C. Helium was used as carrier gas (1ml/min). The relative amount of individual components of the total juice expressed as percentage peak area relative to total peak area. Quantitative identification of the different constituents was performed by comparison of their relative retention times and mass spectra with those of authentic reference compounds, or by retention indices (RI) and mass spectra.(Table 3).

**RESULT AND DISCUSSION**

The combination of five probiotics for diarrhoea causing test pathogen (Tabel 2). Result of our study demonstrated that from 23 trials of probiotic combination the 20th combination (i.e $T_{23}$ . 1+4+5) *Lactobacillus kefiranofaciens+ Candida kefir+ Saccharomyces boluradii* was excellent probiotic approachment for all the five test diarrhoea causing pathogen. The $T_{23}$ probiotics used for fermentation of five different milk samples. This is compare with earlier observations like combination of both *Lactobacillus acidophilus* and *Bifidobacterium bifidum* for probiotic ice cream preparation for inhibitory toward gram negative bacteria (Rasic 1983). However (Michael *et al.,* 2007) recorded probiotics of lactobacillus sp , bifidobacteria as well as probiotic yeast *saccharomyces boulardii* have been investigated with medical use, either as single strains or in mixed cultures.

Comparision of five milk beverages (A1- cow milk, A2- Buffalo milk, A3- Lactogen, A4-NAN, A5- Hatsun) for inhibition of bacterial growth (Table 3). On the basis of result fermented cow milk beverage have the greater antidiarrhoeal effect in all the test pathogen compare with other milk beverage. The proceeding studies have shown protective and therapeutic effect of synbiotic milk beverages to diarrhoeal causing organisms.

In addition to that significant in antagonistic effect against the test pathogen between non fermented milk beverage (S1) , fermented milk beverage (S2), and fermented milk beverage with inulin (S3) was performed (Table 4 & Fig 1). However among the three samples higher antagonistic activity was shown by fermented cow milk beverage with inulin (S3). Similar incidence was reported as the probiotic *Lactobacillus acidophilus* with prebiotic inulin at different concentration (0-5%) results in good probiotic-prebiotic combination for the preparation of synbiotic yoghurt (Anju Kurien *et al.,* 2005).

The report of chromatograms and compounds from fermented cow milk beverage given in (Table 5 & Fig 2). Chromotographic analysis of compounds obtained from the milk beverage of fatty acid, palmitic acid, stearic and the unsaturated acids, ollic besides metalinic acids, fatty oils of fermented milk beverage can be used as natural anti bacterial potencial activity after further studies.

**Table 1: Identification of Probiotic organisms**

| Characteriistics | *L.kefirano faciens* | *L.mesenteroides* | *L.bulgaricus* | *Candida kefir* | *S.boluradii* |
|---|---|---|---|---|---|
| **Cellwall** | G+ve | G+ve | G+ve | Chitin mannose PPM, PLM | Chitin mannose PPM, PLM |
| **Morphology** | Rod | Cocci | Rod | Yeast like pseudohyphae | Pseudohyphae |
| **Motility** | NM | NM | NM | - | - |
| **Spore forming** | NS | - | NS | | |
| **Selective medium** | MLR | TJA | LBB | YMA | SGA |
| **Growth at** | | | | | |
| $15^0$C-$20^0$C | + | + | + | + | + |
| $20^0$C -$30^0$C | | | | | |
| $30^0$ C-$40^0$C | | | | | |
| $40^0$ C-$50^0$C | | | | | |
| **PH** | | | | | |
| 3.5 | + | + | + | + | + |
| 4.5 | | | | | |
| 6.5 | | | | | |
| 8.5 | | | | | |
| **Salt** | | | | | |
| 6.5% | + | + | + | + | + |
| 10% | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| **Carbo hydrate fermentation** | | | | | |
| Arabinose | + | + | + | + | + |
| Cellobiose | + | + | + | + | W |
| Esuculin | + | - | + | + | - |
| Fructose | + | W | + | + | + |
| Galactose | + | + | W | + | + |
| Gluconicacid | + | + | + | - | + |
| Lactorose | + | + | - | + | + |
| Maltose | + | + | + | - | + |
| Mannitol | + | + | + | - | + |
| Mannose | + | + | + | - | + |
| Mellibiose | - | + | + | + | + |
| Raffinose | - | + | + | - | + |
| Rhamnose | + | + | + | - | + |
| Ribose | + | + | + | - | + |
| Salicin | + | + | + | - | + |
| Sorbitol | + | + | + | - | - |
| Sucrose | - | + | + | - | + |
| Xylose | | + | + | - | + |

(++) - Luxurious growth

(+) - growt

(W) - Weak Growth

(-) - No growth

PPM – Phophopetidomannan

PLM – Phospholipomannam

MLR – Modified Lactobacillus Agar medium

TJA – Tomato Juice Agar medium

LBB – Lacto bacillus bulgaricus agar medium

YMA – Yeast morphology agar medium

SGA – Sabrouds glucose medium

NM – Nonmotile

NS - Nospore

**Table 2: Combinational Approachment of probiotics against diarrhoeal causing organisms**

| **Organism** | **$T_1$ (1)** | **$T_2$ (2)** | **$T_3$ (3)** | **$T_4$ (4)** | **$T_5$ (5)** | **$T_6$ (1+2)** | **$T_7$ (1+3)** | **$T_8$ (1+4)** | **$T_9$ (1+5)** | **$T_{10}$ (2+3)** | **$T_{11}$ (2+4)** | **$T_{12}$ (2+5)** | **$T_{13}$ (3+4)** | **$T_{14}$ (3+5)** | **$T_{15}$ (1+2+3)** | **$T_{16}$ (1+2+4)** | **$T_{17}$ (1+2+5)** | **$T_{18}$ (1+3+4))** | **$T_{19}$ (1+3+5)** | **$T_{20}$ (1+4+5)** | **$T_{21}$ (2+3+4)** | **$T_{22}$ (2+3+5)** | **$T_{23}$ (3+4+5)** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Staphylococcus auroeus* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ++ | + | + | + |
| *E.Coli* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ++ | + | + | + |
| *S.Paratyphi A* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ++ | + | + | + |
| *Shigella Dysentriae* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ++ | + | + | + |
| *Vibrio Cholerae* | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | ++ | + | + | + |

$T_1$-$T_{23}$ - Trials of probiotic combinations

*1* - *Lactobacillus Kefirano Faciens*

*2* - *Leuconostoc Mesenteriodes*

*3* - *Lactobacillus Bulgaricus*

*4* - *Candida Kefir*

*5* - *Sacharomyces Boluradii*

**Table 3: Antagonositic activity of test pathogens in five Milk samples**

| Milk samples | Zone of inhibition (mm) | | | | |
|---|---|---|---|---|---|
| | *Staphylococus aureus* | *Escheria coli* | *Salmonella paratyphi A* | *Shigella dysenteriae* | *Vibrio cholerae* |
| Cow milk | 19 | 20 | 19 | 18 | 18 |
| Buffalo milk | 12 | 12 | 11 | 10 | 10 |
| Lactogen | 13 | 14 | 12 | 12 | 12 |
| NAN | 16 | 15 | 14 | 15 | 15 |
| Hatsun | 15 | 16 | 17 | 15 | 16 |

**Table 4: Inhibitory Activity of Synbiotic Cow Milk Beverage Against Test Pathogens**

| Pathogens | $S_1$ | $S_2$ | $S_3$ |
|---|---|---|---|
| *Staphylococus aureus* | + | ++ | +++ |
| *Escheriacoli* | + | ++ | +++ |
| *Salmonella paratyphi A* | + | ++ | +++ |
| *Shigella dysenteriae* | + | ++ | +++ |
| *Vibrio cholerae* | + | ++ | +++ |

$S_1$ - Non fermented cow Milk

$S_2$ - fermented cow Milk

$S_3$ - Fermented cow Milk with Inulin

( + - >1 to10, ++ - 10 to 20 and +++ - > 20 to 30)

**Table 5: Compounds Identified From The Fermented Cow Milk Beverage**

| No | Retention time (min) | Name of the compound | Molecular formula | Molecular weight | Peak area % |
|---|---|---|---|---|---|
| 1 | 8.81 | 1-Hexadecane | $C_{16}H_{32}$ | 224 | 8.89 |
| 2 | 10.36 | Phenol, 2,4-bis (1,1 dimethylethyl) | $C_{14}H_{22}O$ | 206 | 14.90 |
| 3 | 11.22 | 1-Hexadecanol | $C_{16}H_{34}O$ | 242 | 13.62 |
| 4 | 13.72 | 1-Heptadecanol | $C_{17}H_{36}O$ | 256 | 17.13 |
| 5 | 16.31 | n-Hexadecanoic acid | $C_{16}H_{32}O_2$ | 256 | 8.98 |
| 6 | 16.54 | 1-Nonadecene | $C_{19}H_{38}$ | 266 | 14.54 |
| 7 | 18.92 | 9,12-Octadecadienoic acid (Z,Z) | $C_{18}H_{32}O_2$ | 280 | 11.53 |
| 8 | 19.51 | 1-Docosene | $C_{22}H_{44}$ | 308 | 10.41 |

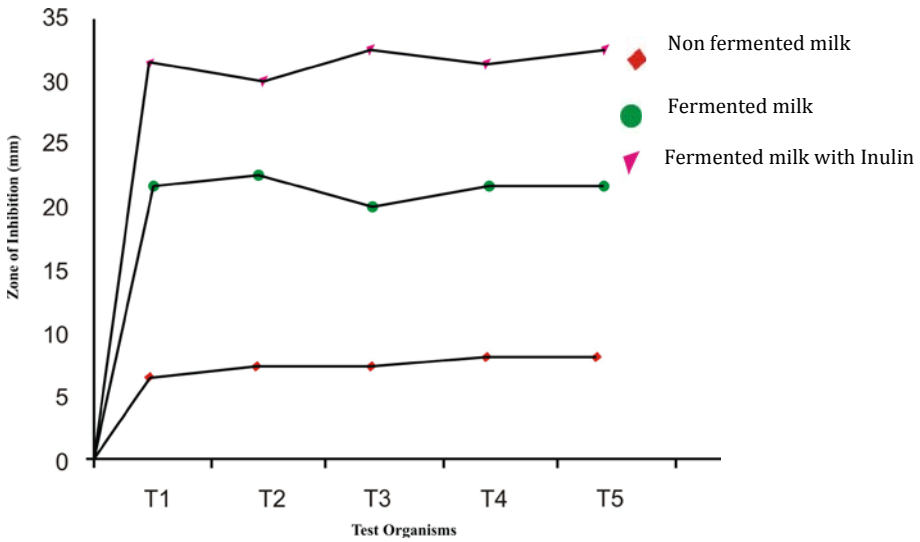


**Fig. 1: Effect On Synbiotic Milk Beverage Against Pathogens**

T1 – *Staphylococcus aureus*

T2 – *E.Coli*

T3 – *Salmonella Paratyphi A*

T4 – *Shigella dysenteriae*

T5 – *Vibrio Cholerae*

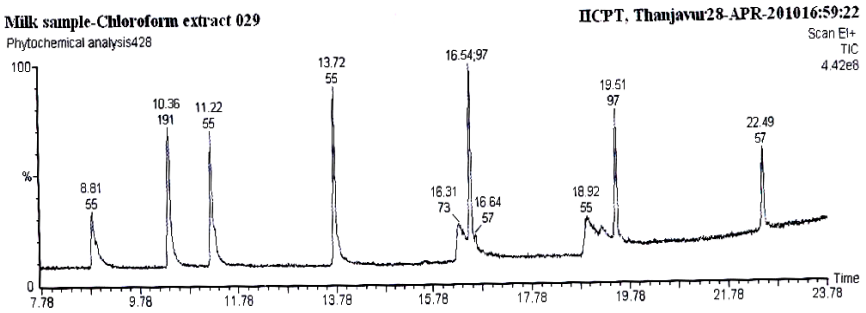


**Fig. 2: Gas Chromatography – Mass Spectrum Analysis of Cow Milk Beverage**

**REFERENCES**

1. Al-Delaimy, K.S. and Ali, S.H. 1970. Antimicrobial action of vegetable extracts on the growthof pathogenic bacteria. *Journal of the science of Food and Agriculture*. 21:110-112.
2. Anju Kurien, A.K., Puniya and Kishan singh. 2005. Selection of prebiotic and *Lactobacillus acidophilus* for symbiotic yoghurt preparation. *Indian Journal of Microbiology.* 45(1):45-50.
3. Ayad, E.H.E., Nashat, S., El-sadek, N., Metwaly, H. and El-soda, M. 2004. Selection of wild lactic acid bacteria isolated from traditional Egyptian dairy product according to production and technological criteria. *Food microbiology*. 21:715-725.
4. Banajeh, S.M., Ba-Oum, N.H. and Al – Sanabani, R.M, 2001. Bacteria aetiology and anti-microbial resistance of childhood diarrhea in Yemen. *J. Trop. Pediatr.* 47:301-303.
5. Chung, K.T., Thomarson, W.R. and Wu Tuan, C.D. 1990. Growth inhibition of selected food borne bacterial *particularia listeria* monocytogens by plate extracts. *J.Appl. Bacteriology.* 69:498-509.
6. Dunne, C.O., Mdhony, L., Murphy, L., Thornton, G., Morrissey, D., Halloran, S., Feeny, M., Flynn, S., Fitzgerald, G., Daly, C., Kiely, B., Sullivan, G.C., Shanahan, F. and Collins, J.K. 2001. In vitro selection criteria for probiotic bacteria of human origin: correlation with in vivo findings. *American Journal of Clinical Nutrition.* 73:386s-392s.
7. El-Naggar, M.Y.M. 2004. Comparative study of probiotic cultures to control the growth of *Escherichia coli* 0157:H7 and *Salmonella typhimurium. Biotechnology*. 3:173-180.
8. Gibson, G.R., Beatty, E.R., Wang, X., Cummings, J.H. 1995. Selective stimulation of *bifidobacteria* in the human colon by oligofructose and inulin. *Gastroenterology.* 108:975-982.
9. Gibson, G.R., Roberfroidl, M.B. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J.Nutr*. 125:1401-1412.
10. Ito, S., Sato, H.; Kudo, S., Sato, H., Nakajima and Toba, T. 2003. The screening of hydrogen peroxide producing lactic acid bacteria and their application to inactivating Psychrotrophic food borne pathogens. *Curr. Microbiology*. 47:231-236.
11. Kahali, S., Salkar, B., Rajendran, K., Khanan, J., Yamasaki, S., Nandy, R.K., Bhattacharya, S.K. and Ramamurthy, T. 2004. Virulence characteristics and molecular Epidemiology of enteroaggressive *Escherichia coli* isolated from hospitalized diarrhoeal patients in kolkata. Ind. J. Clinl. Microbiol. 42:4111-4120.
12. Lankaputhra, W.E.V. and Shah, N.P. 1995. Survival of *Lactobacillus acidophilus and*
13. *Bifidobacterium* spp in the presence of acid and bile salts. *Cultured Dairy Prod. J*. 30:2-7.
14. Lourens – Hattingh, A., Viljoen, B.C. 2001. Yoghurt as probiotic carrier food. *Int. Dairy J.* 11:1-17.
15. Lu, I. and Walker, W.A. 2001. Pathologic and physiologic interaction of bacteria with the gastrointestinal epithelium. *AMJ. Clin. Nutr*, 73:11245-11305.
16. Rasic, J.L. 1983. The role of dairy foods containing bifidobacteria and acidophilus bacteria in nutrition and health. *J. North .Eur. Dairy*. 4:1.
17. Tannock, G.W., Munro, K., Harmsen, H.J., Welling, G.W., Smart, J., Gopal, P.K. 2000. Analysis of the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. *Appl.Environ. Microbiol.* 66:2578-2588.
18. Yoon ky, Wooolams, E., Hang, Y.D. 2006. Production of probiotic plums juice by lactic acid bacteria. J. *biortech*. 97 (12): 1427-30.