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Full Length Research Paper

Effect of some probiotics on *Salmonella typhi* during associated growth in milk

Monadi A. R. [1], Mirzaei H.[2]*, Javadi A.[2], Hosseinzade N. [3] and Amjadi Y.[4]

[1]Department of Microbiology, Faculty of Veterinary Medicine, Islamic Azad University, Tabriz Branch, Tabriz, Iran.
[2]Department of Food Hygiene, Faculty of Veterinary Medicine, Islamic Azad University, Tabriz Branch, Tabriz, Iran.
[3]Agricultural Biotechnology Research Institute of Iran, Northwest and West Region, Tabriz, Iran.
[4]Department of Veterinary Medicine, Islamic Azad University, Tabriz Branch, Tabriz, Iran.

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In this investigation, the effects of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium angulatom* and *Bifidobacterium bifidum* on *Salmonella typhi* was evaluated in associated growth condition in milk. According to statistical tests, in 24 and 48 h incubation of associated growth, *L. acidophilus*, *L. casei* and *B. bifidum* had significant inhibitory effect on the growth of *S. typhi* (p<0.01). But in similar condition, associated growth of *B. angulatum* did not show significant inhibitory effect on *S. typhi* growth. Meanwhile, in 24 and 48 h incubation of associated growth, *L. acidophilus*, *L. casei* and *B. bifidum* significantly reduced (p<0.01) the mean pH of milk samples compared to the control sample. These results demonstrate that the consumption of probiotic products containing *B. bifidum*, *L. acidophilus* and *L. casei* could have benefical effects in prevention of *S. typhi* caused infections. Though, further research especially at *in vivo* condition should be carried out in this field.

Key words: *Salmonella typhi*, probiotics, associated growth, milk.

INTRODUCTION

The genus *Salmonella* belongs to the family Enterobacteriaceae. Like other Enterobacteriaceae genera, *Salmonella* is gram-negative flagellated rode-shaped bacterium. *Salmonellae* are facultative anaerobes with both respiratory and fermentative metabolic pathways. They are oxidase negative, ferment glucose and produce acid and gas (Yousef and Carlstrom, 2003).

There are three host adaptation patterns among the *Salmonella* serovars. Human host adapted serovars tend to cause the most severe illness. The most highly host-adapted organism of interest in human medicine is *Salmonella typhi*, the cause of human typhoid fever. There is no animal source of *S. typhi*. Therefore, *S. typhi* is always contracted from water or food that has been contaminated in some manner by another human being. They are very rare exceptions to human-to-human transmission which occur as laboratory accidents (Ziprin and Hume, 2001).

Human digestion system is natural primordium of massive and dynamic population of bacteria (Guarner and Malagelada, 2003). This complex ecosystem contains more than 500 species (Blaut et al., 2002) and immense information had been achieved about important role of these bacteria in health preservation and disease prevention (Holzapfel et al., 1998; Marteau et al., 2002). Destruction of colon flora by pathogens, nutritional antigens or other deleterious materials can lead to functional disorder of colon (Bartlett et al., 1987; Siitonen et al., 1990). Lactobacilli and bifidobacteria with their long history in dairy products, have been traditionally utilized in probiotic products for preservation against such destructive effects. Lactobacilli set in digestion system immediately after human babe birth. In healthy people lactobacilli are naturally present in mouth area (10^3 to 10^4cfu/g), ileum (10^3 to 10^7 cfu/g) and colon (10^4 to 10^8 cfu/g), also they are dominant microorganisms in vagina (Hill et al., 1984). According to several studies, lactobacilli produce a vast range of antibacterial compounds including oxygen catabolites such as hydrogen peroxide, sugar catabolites such as organic acids (e.g., lactic acid and acetic acid); proteinaceous compounds such as bacteriocins, other low-molecular-mass peptides, and antifungal peptides/proteins; fat and amino acid metabolites such as fatty acids, phenyllactic acid, and

OH^- phenyllactic acid, and other compounds such as reuterin and reutericyclin (Alakomi et al., 2000; Ogawa et al., 2001; Servin, 2004; Valerio et al., 2004; Makras et al., 2006).

Bifidobacteria are part of natural flora of human colon which exist in comparatively high numbers (10^9 to 10^{10} cfu/g) (Macfarlane and Macfarlane, 2003). The data revealed by different researchers had confirmed the beneficial effects of bifidobacteria (Gomes and Malcata, 1999; Salminen et al., 1996; Salminen et al., 1988). These bacteria help to the host health via balancing intestinal microbes, fermentation of indigestible oligosaccharides which are not absorbed in small intestine or by affecting other bacteria. According to several studies, inhibition of vast variety of pathogenic microorganisms using bifidobacteria has been proved at *in vivo* and *in vitro* conditions. Several mechanisms have been suggested for inhibitory function of bifidobacteria against gram-negative pathogens (Fooks and Gibson, 2002; Fujiwara et al., 1997; Servin, 2004).

In this study, the effects of four probiotic bacteria including *Bifidobacterium angulatum*, *Bifidobacterium bifidum*, *Lactobacillus acidophilus* and *Lactobacillus casei* on growth rate of *S. typhi* was studied in milk which is an excellent medium for growth of many micro-organisms during 24 to 48 h incubation in 37°C (human body temperature).

MATERIALS AND METHODS

Bacterial strains and media

Lactose broth, peptone water, MRS (Man-Rogosa-Sharp) Agar, Nutrient Agar and *Salmonella Shigella* agar (Merk) were used as culture media. Furthermore the strains of *B. angulatum* PTCC 1366, *B. bifidum* PTCC 1644, *L.acidophilus* PTCC 1643 were obtained from Persian Type Culture Collection (PTCC). Lactic starter containing the strain *L. casei* 01 (Hansen CHR) and the strain *S. enteric* serovar typhi were prepared from 'Pak' milk factory and microbiology laboratory in medicine faculty of Tabriz medical science university, respectively.

Study design

According to suggestion of the provider center, lyophilized probiotic strains were inoculated in erlenmeyers containing 100 ml peptone water and the strain *S. typhi* was inoculated in erlenmeyer containing 100 ml lactose broth. These cultures were incubated for 24 h at 37°C. Then for the formation of colonies, activated probiotics of 4 erlenmeyers and activated *S. typhi* were cultured in 4 plates of MRS agar and one plate of nutrient agar by surface plate method. The cultures were incubated for 24 TO 48 h at 37°C. Afterwards, 1.5×10^8 cfu of *S. typhi* was inoculated in 500 ml of sterile milk and after 15 min of homogenizing was equally distributed in 5 sterile 100 ml erlenmeyers. The first erlenmeyer was considered as control sample (monoculture). To the second, third, fourth and fifth flaskes, 1.5×10^8 cfu of above-mentioned probiotics were inoculated respectively. These erlenmeyers were incubated for 24 to 48 h at 37°C and pH of the samples was measured by pH meter with a glass electrode (632 pH Meter, Metrohm Herisau, Switzerland). In the first 24 h after incubation and the second 24 h respectively, up to 10^{-7} and 10^{-6} serial dilutions were prepared from 5 Erlenmeyer contents. Dilutions of 10^{-5} to 10^{-7} in the first 24 h and of 10^{-4} to 10^{-6} in the second 24 h after incubation were cultured in SSA by pour plate method. Then plates were incubated for 24 h at 37°C. Plates containing 30 to 300 colonies were used for colony counting and calculating the number of *S. typhi* in each milliliter of the content (Karim, 2002). The mean pH and number of *S. typhi* (cfu/ml) of above-mentioned culture contents were analyzed using statistical tests of one way analysis of variance and Tukey analysis. In order to calculate the inhibitory effect rate of each probiotic on *S. typhi* (S) growth, below written formula was applied.

$$\% \text{ Inhibition of S} = \frac{\text{Count of S in monoculture - count of S in associated culture}}{\textit{Count of S in monoculture}} \times 10$$

RESULTS

The results of effect of each aforesaid probiotic on the number of *S. typhi* and pH of milk in associated growth condition are depicted in Tables 1 and 2.

According to the obtained results, associated growth of *L.acidophilus*, *L. casei*, *B. angulatum* and *B. bifidum* in 24 h inhibited 54.1, 32.23, 29.41 and 35.44% of *S. typhi* growth rate, respectively; while in 48 h the inhibited rates were as 63.13, 73.7, 48.73 and 66.49%, respectively.

DISCUSSION

This study shows that *L. casei* and *L. acidophilus* have significant inhibitory effect on *S. typhi* ($p<0.01$); the environmental pH reduction could be considered as one factor of the inhibitory effect. It has totally been accepted that weak organic acids such as lactic acid show potent antibacterial function (Alakomi et al., 2000). Lactic acid present in human colon is in low concentration. This acid is produced as intermediate product in fermentation of carbohydrates. In colon, it is converted to short-chain fatty acids which could be in the concentration of above 100 mM. Therefore, lactic acid can act as one of antibacterial factors against gram-negative pathogens (Duncan et al., 2004; Macfarlane and Macfarlane, 2003, Topping and Clifton, 2001).

Only in a limited number of reports, organic acid production had been suggested as the main factor in the function of Lactobacilli against gram-negative bacteria (Gagnon et al., 2004; Ogawa et al., 2001). On the contrary, in a lot of researches, production of other antibacterial compounds is considered as the cause of Lactobacilli activity against *Salmonella* and *E. coli* (Servin, 2004).

It had already been pointed that inhibitory materials such as hydrostatic and aromatic molecules e.g. mevalonolactone which are produced by *L. plantarum*, act against gram-negative bacteria only in low pH range and in the presence of lactic acid. More importantly, lactic acid plays its role via making gram–negative bacteria's wall permeable and it causes antibacterial activity of

Table 1. The number (cfu/ml) of *S.typhi* in monoculture and associated cultures after 24 and 48 h of incubation.

Cultures	Number of *S.typhi* (cfu/ml)							
	After 24 h incubation				After 48 h incubation			
	N	Mean	SD	SE	N	Mean	SD	SE
S. typhi	10	305.3×105	47.1×104	14.9×104	10	332.8×104 a	238.6×103	75.4×103
S. typhi +B. angulatum	10	215.5×105 ab	77.5×104	24.5×104	10	170.6×104 ab	156.4×103	49.4×103
S. typhi +B. bifidum	10	197.1×105 ab	99.6×104	31.5×104	10	111.5×104 b	41.5×103	13.1×103
S. typhi + L. acidophilus	10	176.7×105 b	109.7×104	34.7×104	10	122×104 b	62.1×103	19.63×103
S. typhi + L. casei	10	139.8×105 b	96.5×104	30.5×104	10	87.5×104 b	65.9×103	20.8×103

N: Number, SD: Standard deviation, SE: Standard error of mean. a and b: Means along the column with different superscript are significantly different (P <0.01).

Table 2. pH of monoculture and associated cultures after 24 and 48 h of incubation.

Cultures	pH							
	After 24 h of incubation				After 48 h of incubation			
	N	Mean	SD	SE	N	Mean	SD	SE
S. typhi	0	5.863[a]	0.151	0.047	10	5.596[a]	0.205	0.064
S. typhi +B. angulatum	10	5.620[b]	1.199	0.063	10	5.269[ab]	0.273	0/086
S. typhi +B. bifidum	10	5.620[b]	0.219	0.069	10	5.050[b]	0.415	0.131
S. typhi + L. acidophilus	10	5.451[b]	0.170	0.053	10	4.930[b]	0.324	0.102
S. typhi + L. casei	10	Mean	0.193	5.469[b]	10	4.936[b]	0.337	0.106

N: Number, SD: Standard deviation, SE: Standard error of mean. a and b: Means along the column with different superscript are significantly different (P <0.05).

other inhibitory compounds (Alakomi et al., 2000). Phenyllactic acid and OH^- phenyllactic acid are also broadly produced by lactobacilli and show wide spectra of antifungal activity in the concentrations of 100 to 400 µM (Topping and Clifton, 2001; Valerio et al., 2004). Moreover, phenyllactic acid shows inhibitory activity against gram-positive bacteria such as *Lysteria monocytogenes*, *Staphylococcus aureus* and enterococcus faecalis, and against gram–negative bacteria such as *Providencia stuartii* and *Klebsiella oxytoca* (Dieuleveux et al., 1998).

According to Table 1, the associated growth of *B. bifidum* after 48 h of incubation has significant inhibitory effect on *S. typhi* ($p<0.01$) and based on the data in Table 2, pH reduction can be considered as one factor of this effect.

Gibson and Wang (1994) discovered that the inhibitory feature of *Bifidobacterium infantis* against *E. coli* and *Clostridium perfringens* is not solely related to acid production. Indeed it is suggested that basic metabolites are not only efficient causes in inhibitory function. Other factors including nutritional competition, variation of oxidation-reduction potential, H_2O_2, diacetyl, bacteriocin, bacteriocin-like inhibitory materials produced by some strains of *Bifidobacterium* and *Lactobacillus* are impressive, as well. This has generally been accepted that inhibitory effect of bifidobacteria and lactobacilli is as a result of interaction between these factors.

The production of special antibacterial compounds by bifidobacteria had already been reported. For example, two strains of bifidobacteria have been isolated from baby excretion which has powerful lethal activity against several pathogenic bacteria such as *S. typhimorium* SL1344 and *E. coli* C1845. The lethal effect had been attributed to mass production of potentially low molecular weight and lipophilic molecules. A protein mass with low molecular weight called BIF which is produced by *B. Longum* is the only active compound against gram-negative bacteria that is known so far (Fujiwara et al., 1999; Fujiwara et al., 2001; Fuller, 1989). This protein does not have direct inhibitory or lethal property but it controls the attachment of *E. coli* to epithelial cells in human body. Similarly, production of special antibacterial compounds by bifidobacteria had been suggested in other reports (Gagnon et al., 2004). In other studies, it has been proved that inhibition of *S. enterica typhimorium* SL1344 and C1845 is directly related to organic acid production and environmental pH reduction. Indeed other antibacterial compounds could not be ignored but their proportions in inhibition of the gram-negative pathogens seem trivial. It sounds presence of organic acids is the main mechanism of inhibitory effect of bifidobacteria.

Organic acids enter the bacteria as a whole molecule and are decomposed inside cytoplasm of bacteria. The final reduction of pH inside bacteria cells and accumulation of ionized organic acids inside bacteria lead to death of the pathogen (Reid et al., 2003).

As seen in Table 1, associated growth of *B. angulatum* in spite of *B. bifidum* does not have inhibitory effect on *S. typhi*.

These results were in consistent with other researchers' discoveries that antibacterial function of bifidobacteria is different from one strain to the other (Ibrahim and Bezkorovainy, 1993). It indicates that based on desired criteria, the strain selection of bifidobacteria as probiotic should be performed carefully. Altogether, the results of this research show that if the cells of *L. casei*, *L. acidophilus* and *B. bifidum* be settled in human digestion system via consumption of fine products containing these microorganisms, they can be helpful in prevention of the diseases caused by *S. typhi*. Indeed, further studies are essential to be carried out in this aspect especially at *in vivo* condition.

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