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Interaction of *Bifidobacterium* and Yoghurt Mixed Culture with *Salmonella* During Associated Cultures Growth

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Abstract: The antimicrobial activity of four strains of *Bifidobacterium* toward *Salmonella* ssp. during associated cultures growth was investigated in skim milk medium. All strains showed different degrees of antagonistic action toward the indicator strain. The highest degree of inhibition (96%) was obtained with *Bifidobacterium infantis* and *Bifidobacterium longum* (92%). The combination effect of yogurt mixed culture with bifidobacterial strains (di-associated culture) toward *Salmonella* ssp. resulted in an enhancement of the antagonistic action for *Bifidobacterium* strains as a result of their production of organic acids, in particular lactic acid, which has a strong inhibitory effect against Gram-negative bacteria. The di-associated cultures all resulted in similar pH values but the degree of inhibition were different with *B. infantis* and *B. longum*; meaning that organic acids are not the sole inhibitory factors present in these cultures, but it could be another compounds which may contribute in this inhibitory effects. The combination between *Bifidobacterium* and YMC strains could has a great value in industrial application in resolving some problems in dairy products and pharmaceutical formulas.

Key words: *Bifidobacterium*, associated cultures, degree of inhibition, di-associated cultures, yogurt mixed culture

INTRODUCTION

The human endogenous intestinal microflora plays an important role in providing nourishment, regulating epithelial development and instructing innate immunity (Eckburg *et al*., 2005). In the normal gut the relationship between the microbiota and the host is mutually beneficial of which the microbiota is provided with steady growth conditions and some nutrient supply. In return, the microbiota contributes to the host's nutrition, immune system development, angiogenesis and fat storage (Cash *et al*., 2006). This complex network of interactions is thought to stabilize the population structure of the microbiota and to prohibit colonization by intruding pathogens (Stecher and Hardt, 2008). Bifidobacteria is one of the first bacteria to colonize the gastrointestinal tract immediately after birth with some other bacteria include aerobic and anaerobic bacteria, such as *Escherichia coli*, *Clostridium* spp., *Streptococcus* spp., *Lactobacillus* spp. and *Bacteroides* spp. (Servin, 2004).

Probiotic bacteria are defined as live microorganisms that when administered in adequate amounts confer a health benefit on the host (FAO, 2001). Due to their established health benefits, probiotic bifidobacteria and lactobacilli are increasingly incorporated into foods, mainly dairy products (Saarela *et al*., 2000). Health-promoting properties have been demonstrated for specific probiotic products. Bifidobacteria appear to be the most promising probiotic candidates, followed by defined lactic acid bacteria which favor specific healthy bifidobacterial growth and species composition (Salminen and Isolauri, 2006).

The reported health benefits of bifidobacteria include stabilizing the gut mucosal barrier, modulation of immune response, modulation of intestinal microbiota, prevention of traveller's diarrhoea, treatment of viral diarrhoea, alleviation of IBS symptoms in adults, improvement of constipation and antibacterial and anticarcinogenic activities (Gomes and Malcata, 1999; Saarela *et al*., 2000; Ouwehand *et al*., 2002; Reid *et al*., 2003).

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Salmonella is one of the most important foodborne pathogens of public health significance and continues to be a major concern to regulatory agencies and the food industry. The pathogen is a leading cause of gastroenteritis and a variety of foods including meat and poultry, milk, ice cream, cheese, eggs and egg products, chocolate and spices have been implicated as vehicles of transmission (D'Aoust, 1989) for causing salmonellosis. Antagonistic activity of *Bifidobacterium* against *Salmonella* was examined *in vitro* by several researchers in both agar media and in broth media (Bernet *et al*., 1993; Bielecka *et al*., 1998).

Lactic acid bacteria (LAB), which include the genera *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Enterococcus*, *Carnobacterium*, play an essential role in food fermentations given that a wide variety of strains are routinely employed as starter cultures in the manufacture of dairy, meat and vegetable products (Nettles and Barefoot, 1993). The most important contribution of these microorganisms to the product is to preserve the nutritive qualities of the raw material through an extended shelf life and the inhibition of spoilage and pathogenic bacteria such as *E. coli* and *Salmonella*. This is due to competition for nutrients and the presence of inhibitors produced by the starters, including organic acids, hydrogen peroxide and bacteriocins (Ray, 1992).

The aim of this research was to study the antimicrobial effects of *Bifidobacterium* strains on the kinetics of *Salmonella* during associated cultures and also the combination effects with Yogurt Mixed Culture (YMC) and *Bifidobacterium* strains on *Salmonella* ssp. growth kinetics in di-associated cultures.

MATERIALS AND METHODS

The research presented here was conducted on the premises of State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu, China during the year 2007.

Bacterial strains and culture conditions: *Bifidobacterium* strains and *Salmonella* ssp. strain and their culture conditions which were used in this study are shown in Table 1. YMC [MY900 (ST+LB)], which is composed of two strains; *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* obtained from Rhodia (ZA de Buxieres-B.P.10-France). *Bifidobacterium* strains were routinely cultured in MRS broth (De Man *et al*., 1960) obtained from Merck (Darmstadt, Germany) supplemented with 0.05% (w/v) L-cysteine-hydrochloride (Cys-HCl, Sigma, St. Louis, MO, USA) and incubated under anaerobic conditions using an atmosphere generation system (GasPak system, Oxoid, Basingstoke, Hampshire, England) at 37°C. *Salmonella* ssp. was maintained in Nutrient Agar (NA) (Oxoid) with pH 6.8 and enumerated on the same medium. Agar plates and broth (10 mL) inoculated with *Salmonella* strain were incubated at 37°C for 18 h aerobically. YMC was cultured according to the manufacturer's instructions (Rhodia, France).

Table 1: List of microorganisms, sources and their culture conditions

Microorganism*	Source/ strain**	Medium***	Cultivation temp. (°C) and condition
Bifidobacterium infantis	BCRC 14602	MRS-C	37, anaerobic
Bifidobacterium longum	BCRC 14634	MRS-C	37, anaerobic
Bifidobacterium adolescentis	BCRC 14606	MRS-C	37, anaerobic
Bifidobacterium bifidum	BCRC 14615	MRS-C	37, anaerobic
Salmonella enterica ssp. *enterica*	ATCC 13076	NA, NB	37, aerobic
Salmonella typhimurium	ATCC 29631	NA, NB	37, aerobic
Salmonella enteritidis	CMCC(B) 50041	NA, NB	37, aerobic
Salmonella sp.	MLCC	NA, NB	37, aerobic
(YMC) [MY900 (ST+LB)]	Rhodia-France	MRS	30, aerobic

*YMC, Yogurt Mixed Culture; which contains: *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*, **BCRC: Bioresource Collection and Research Center, Food Industry Research and Development Inst., Hsinchu, Taiwan; ATCC, American Type Culture Collection, Manassas, USA; CMCC: China Microbiological Culture Collection, Beijing, China; MLCC: Microbiology Laboratory Culture Collection, Jiangnan University, Wuxi, Jiangsu, China.***MRS-C, deMan Rogosa Sharpe agar supplemented with 0.05% (w/v) cysteine hydrochloride; NA: Nutrient Agar; NB: Nutrient Broth

Growth of *Bifidobacterium* and *Salmonella* ssp. in skim milk: Cultures were done in skim milk medium (Bright Dairy Company, Shanghai, PRC) for the assessment of inhibitory activity of *Bifidobacterium* strains against *Salmonella* ssp. The skim milk was prepared with 12% (w/v) and pH 6.60 and then autoclaved at 115°C for 15 min. The skim milk medium was inoculated simultaneously with *Salmonella* strain (10^6 cfu mL^{-1}) and *Bifidobacterium* strains (10^8 cfu mL^{-1}). The cultures were then incubated for 60 h at 37°C under aerobic conditions. The numbers of colony forming units (cfu mL^{-1}) were determined every 12 h within a 60 h time period using nutrient agar (Oxoid) supplemented with 10 g L^{-1} glucose.

Combination between Yogurt Mixed Culture (YMC) and *Bifidobacterium*: Combinations of YMC and *Bifidobacterium* strains were done for the assessment of their inhibition effects against *Salmonella* ssp. strains by broth culture experiment in skim milk (pH 6.60). Measurements of pH and Total Plate Count (TPC) for *Salmonella* ssp. (Mono-cultures) were done and comparisons with associated cultures (*Salmonella* ssp. strains with *Bifidobacterium* ssp.) and di-associated cultures (*Salmonella* ssp. strains with *Bifidobacterium* ssp. +YMC*)* were performed.

Kinetics and degree of inhibition: The degree of inhibition of *Salmonella* (Sal) was expressed by the following equation:

$$\text{Sal inhibition (\%)} = \frac{[\log \text{cfu mL}^{-1} \text{ Sal MC} - \log \text{cfu mL}^{-1} \text{ Sal DiC}]}{\log \text{cfu mL}^{-1} \text{ Sal MC}} \times 100$$

(DiC) : Di-associated cultures (*Salmonella*+ *Bifidobacterium*+YMC)
(MC) : Monoculture (*Salmonella* ssp.)

Statistical analysis: Each experiment was independently replicated three times in a completely randomized design and the means values were calculated. Standard deviation was also calculated for the replicated values of pH and TPC for *Salmonella* strains and *Bifidobacterium* strains by using the software package Decision Tools Suite Software StatPro TM for Microsoft Excel (New York, USA). Data were subjected to one-way Analysis of Variance (ANOVA) followed by Duncan multiple range test to determine the significance ($p<0.05$) of differences.

RESULTS

Growth properties of *Bifidobacterium* and *Salmonella* ssp.: *Salmonella* spp. numbers in Nutrient broth with pH 6.80 reached up to 8.40 cfu mL^{-1} after 60 h and cultivation medium reached pH of 6.44. In skim milk medium the cfu mL^{-1} number reached up to 9.20 and the pH reached 5.24. Bifidobacteria obtained highest cfu numbers in skim milk medium after 48 h and after 36 h in C-MRS broth (Cheikhyoussef *et al*., 2007). It was observed that bifidobacteria decrease the pH (in skim milk; pH ranged from 6.60 to 3.99 and in MRS from 6.80 to 3.38) of their culture media to a greater extent than the *Salmonella* strains (in skim milk pH ranged from 5.24 to 5.04; in Nutrient Broth pH ranged from 7.4 to 6.8) and this is as a result of their production of organic acids.

Antimicrobial effects of *Bifidobacterium* strains towards *Salmonella* spp. in associated cultures: In associated cultures with *Bifidobacterium* strains in skim milk; the *Salmonella* populations ranged from 0.12 to 0.42 log cfu mL^{-1} after 60 h incubation proving the antimicrobial effects from *Bifidobacterium* strains towards *Salmonella* spp. (Table 2).

The highest degree of inhibition (95.83%) obtained with *B. infantis* (Fig. 1A) and 92.08% for *B. longum* (Fig. 2A). Also the pH was reduced from 5.04 in monoculture to 4.00-4.22 in associated culture after 60 h incubation; this pH reduction is a common result from the bifidobacterial production of organic acids.

Combination effects in di-associated cultures: The combination of YMC with *Bifidobacterium* strains caused the pH to drop only slightly compared to the associated cultures. The TPC numbers of *Salmonella* spp. populations in di- associated cultures were reduced by 95.1% with *B. infantis* and by 83.2 % with *B. longum* after a 12 h incubation period (Fig. 1B, 2B). The di-associated cultures all resulted in similar pH values (Associated culture pH 3.83 compared to 3.72 in di-associated for *B. infantis* and 3.88 to 3.76 for *B. longum*) but the degree of inhibition especially with *B. infantis* and *B. longum* were different suggesting that organic acids are not the sole inhibitory compounds present in these cultures supernatants.

Table 2: Growth and inhibition percentages of *Salmonella* ssp. populations in monoculture, associated and di-associated culture after 60 h incubation

Bifidobacterium strains	*Salmonella* strains	*Salmonella* monocultures (log cfu mL^{-1})*	Associated culture (log cfu mL^{-1})	Di-associated culture (log cfu mL^{-1})	Inhibition (%) associated culture	Di-associated culture
B. adolescentis	*Salmonella* sp.	7.2**±0.31***^{aB}	1.03±0.34^{aA}	0.63±0.34^{aA}	85.64±4.78$^{bA\alpha}$	91.20±6.79$^{bB\alpha}$
	S. enteritidis	8.6±0.22^{aB}	3.12±0.54^{aA}	2.49±0.47^{bA}	63.63±6.35$^{cA\alpha}$	71.04±5.46$^{bB\alpha}$
	S. typhimurium	9.2±0.42^{aB}	3.78±0.61^{bcA}	1.97±0.70^{aA}	58.87± 6.7$^{cA\alpha}$	78.58±7.8$^{aB\alpha}$
	S. enterica ssp. *enterica*	7.8±0.15^{aB}	4.74±0.69^{cA}	2.82±0.47^{bA}	39.14±8.88$^{aA\alpha}$	63.80±6.08$^{aB\alpha}$
B. bifidum	*Salmonella* sp.	7.2±0.31^{aB}	1.46±0.86^{aA}	0.96±0.26^{aA}	79.62±12.04$^{cA\alpha}$	86.40±3.72$^{aB\alpha}$
	S. enteritidis	8.6±0.22^{aB}	3.05±0.94^{aA}	1.93±0.22^{aA}	64.45±10.99$^{cA\alpha}$	77.47±2.63$^{cB\alpha}$
	S. typhimurium	9.2±0.42^{aB}	3.53±0.49^{bA}	2.12±0.73^{aA}	61.55±5.35$^{cA\alpha}$	76.91±8.03$^{bcB\alpha}$
	S. enterica ssp. *enterica*	7.8±0.15^{aB}	3.81±0.55^{bcA}	2.81±0.81^{bA}	51.10±7.06$^{bA\alpha}$	63.88±10.5$^{aB\alpha}$
B. infantis	*Salmonella* sp.	7.2±0.31^{aB}	0.30±0.19^{aA}	0.18± 0.13^{aA}	95.83±8.30$^{hA\alpha}$	97.50±4.46$^{gA\alpha}$
	S. enteritidis	8.6±0.22^{aB}	2.01±0.80^{aA}	1.51±0.75^{aA}	76.58±9.30$^{eA\alpha}$	82.36±8.83$^{dB\alpha}$
	S. typhimurium	9.2±0.42^{aB}	2.79±0.62^{aA}	2.02±0.22^{aA}	69.63±6.73$^{dA\alpha}$	78.03±2.48$^{cB\alpha}$
	S. enterica ssp. *enterica*	7.8±0.15^{aB}	3.43±0.33^{bA}	2.21±0.39^{bA}	56.02±10.32$^{cA\alpha}$	71.66±5.81$^{bB\alpha}$
B. longum	*Salmonella* sp.	7.2±0.31^{aB}	0.57±0.27^{aA}	0.29±0.19^{aA}	92.08±7.95$^{gA\alpha}$	95.97±2.65$^{gB\alpha}$
	S. enteritidis	8.6±0.22^{aB}	1.92±0.70^{aA}	1.46±0.69^{aA}	77.59±8.21$^{eA\alpha}$	83.02±8.09$^{dB\alpha}$
	S. typhimurium	9.2±0.42^{aB}	2.83±0.28^{aA}	1.86±0.35^{aA}	69.16±3.10$^{eA\alpha}$	79.78±3.84$^{cB\alpha}$
	S. enterica ssp. *enterica*	7.8±0.15^{aB}	3.14±0.20^{aA}	2.06±0.63^{aA}	59.74±2.56$^{dA\alpha}$	73.54±8.08b○B○

*CFU, colony forming unit; **Values are the mean values from three replicates; ***Standard deviation; Values with different lowercase letter(s) (a-c) in the same column different significantly ($p<0.05$). Values with different uppercase letters (A-C) in the same row different significantly ($p<0.05$); Values with different lowercase letter(s) (a○-f○) in the same inhibition column differ significantly ($p<0.05$); Values with different lowercase letter(s) (A○-B○) in the same inhibition column different significantly ($p<0.05$)

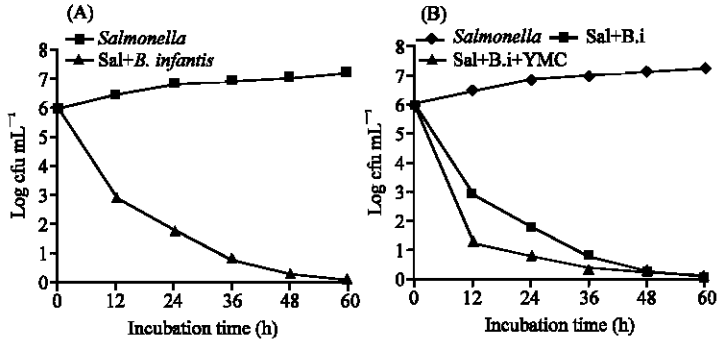

Fig. 1: The antimicrobial effects of *Bifidobacterium infantis* on the kinetic growth of *Salmonella* during associated (A) and Di-associated (B) culture after 60 h incubation

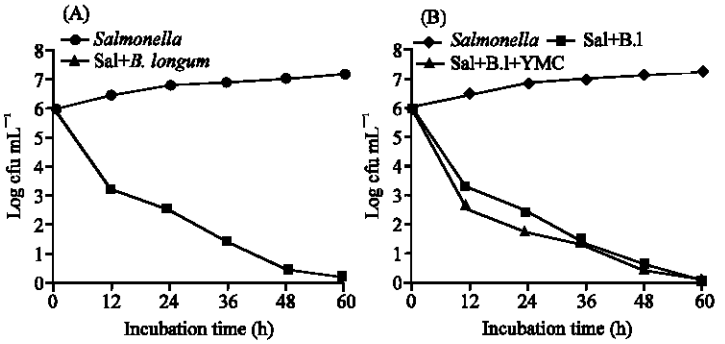

Fig. 2: The antimicrobial effects of *Bifidobacterium longum* on the kinetic growth of *Salmonella* during associated (A) and Di-associated (B) culture after 60 h incubation

DISCUSSION

It is well known that the major metabolites of bifidobacteria are acetic acid and lactic acid in ratio 3:2 and this production is responsible for the associated drop in pH, which is responsible for the antagonism of many pathogenic bacteria belonging to both Gram-positive and Gram-negative bacteria (Rioradan and Fitzgerald, 1998; Makras and De Vuyst, 2006).

The low pH values of the *Bifidobacterium* cultures did not inhibit their growth showing their resistance to an acidic environment. This is an important property for probiotic strains, which enables them to be probiotic carriers passing through the gastrointestinal tract and ending up in the large intestine in the required numbers to provide a probiotic effect against pathogenic bacteria such as *Salmonella enterica* ser. *typhimurium* (Bielecka *et al*., 1998; Liévin *et al*., 2000; Collado *et al*., 2005).

The pH values in associated and di-associated cultures were similar but the degree of inhibition was different; this indicates that there are other factors contributing to this inhibition effects. This data is in agreement with those obtained by Gibson and Wang (1994) who reported that the inhibitory effect of *B. infantis* against *Escherichia coli* and *Clostridium perfringens* were not necessarily attributed to acid production but it's attributed to the production of a potential low-molecular-mass, lipophilic molecule. Results are also in agreement with Trejo *et al*. (2006); who reported on the production of inhibitory substances against *C. difficile* other than organic acids by *Bifidobacterium* 5311 and 532. These two strains have lower concentrations of non-dissociated lactic and acetic acids compared to the high acid production strains like *Bifidobacterium* strains 5319 and 5316 but had the same inhibition strength. Mean while these results are not in agreement with the previous studies made by Makras and De Vuyst (2006) who concluded that the antimicrobial activity of bifidobacterial strains (*B. longum* CA1) on *Salmonella enterica* ser. *Typhimurium* SL1344 is pH dependent because when the Cell Free Supernatants (CFSs) of *Bifidobacterium* strains or control medium was adjusted to pH 6.5, no killing effect was obtained.

The acceleration of the death ratio for *Salmonella* spp. population after 12-36 h incubation period compared to the associated cultures is a good result of the combination between *Bifidobacterium* and YMC strains.

This observation is in agreement with combined cultures of bifidobacteria and *L. acidophilus* or other lactic acid bacteria, viz. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, *S. thermophilus* alone or mesophilic aromatic cultures. These combinations have also been advocated as a solution for many problems since there is an increased growth rates (Gomes *et al*., 1998), a reduction of fermentation time (Hoier, 1992), absence of certain sensory and texture defects (Gomes *et al*., 1995) and a further improvement of nutritional value of `bifidus' products (Samona *et al*., 1996).

In conclusion, the results presented here provide evidence that some *Bifidobacterium* strains exert antimicrobial activity against pathogenic bacteria like *Salmonella typhimurium* and this production seems to be varied among *Bifidobacterium* species. The combination of Yoghurt Mixed Culture with *Bifidobacterium* strains caused the pH of the culture to drop; which caused a high death ratio for the *Salmonella* spp. population. Such combinations between *Bifidobacterium* species and some lactic acid bacteria have great advantages in increasing the protective effects of bifidobacteria for the gastrointestinal tract against enteric pathogens also to ensure the safety and quality of many dairy products.

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