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Relationships Between Bile Tolerence and Deconjugation Activity of Bifidobacterium Spp

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Abstract

The degree of bile tolerance and their action on conjugated bile salts were studied on six different strains of Bifidobacteria. They were *Bifidobacterium infantis* (ATCC 27920), *B. adolescentis* (ATCC 11146), *B. breve* (ATCC 15698), *B. bihdurn* (ATCC 35914) and *B. longum* (ATCC 15708). Sodium taurocholate was used as conjugated bile salt. The ability to grow in the presence of 0.3 percent bile salt varied among the six strains tested. *B. longum* (ATCC 15708) was the most tolerant strain.TPY broth containing 0.05 percent of sodium taurocholate was used to measure the deconjugation activity. All bifidobacteria tested were able to deconjugate conjugated bile salt but at various degrees. *B. infantis* (ATCC 27920) showed the highest deconjugation activity, where the cholic acid released was 0.015 μmol/ml.

Introduction

Gilliland (1979) listed several desirable characteristics for an organism to be selected as a probiotic. One of the important characteristics, is their ability to deconjugate bile salts. Bile salts are synthesised in the liver using cholesterol as the substrate. High level of serum cholesterol has been associated with coronary heart disease (CHD). Dietary adjuncts had been described as having potential to decrease serum cholesterol level, thereby reducing the risks of coronary heart disease.

The primary bile acids synthesis in the liver are cholic acid and chenodeoxycholic acid. They were conjugated with glycine and taurine (ratio 3:1) by peptide (amide) linkage to the carboxyl group, forming glycocholic acid and taurocholic acid or glycochenodeoxycholic and taurochenodeoxycholic acids, respectively. (Midtvedt and Norman, 1972). Conjugation of bile acids 'occurs' in the liver (Ganong, 1981). Conjugated bile acids aids in lipids digestion and absorption by forming polymolecular aggregates (micelles) with water-insoluble lipids.

In healthy individuals, deconjugation takes place during the enterohepatic circulation. This reaction is catalysed by hydrolase enzyme produced exclusively by intestinal bacteria. Deconjugation of bile acids is important in controlling serum cholesterol concentrations, since deconjugated bile acids do not function as well as conjugated bile acids in the solubilization and absorption of lipids. They were excreted more rapidly than conjugated bile acids. In addition, deconjugated bile acids adhere to bacteria or dietary fibre, thus enhancing excretion of bile acids.

Cholesterol is a precursor of bile acids, the synthesis of new bile acids from cholesterol has the potential to reduce total serum cholesterol concentration. Furthermore, cholesterol absorption into the blood from the intestine is not supported as well by deconjugated bile acids as it is by conjugated form (Buck and Gilliland, 1994).

Bifidobacteria deconjugate bile salts and liberate bile acids, cholic and chenodeoxycholic acid. Bifidobacteria are sensitive to high levels of bile acids. It has been shown that 0.02-0.05 percent chenodeoxycholic acid is bacteriostatic to bifidobacteria. At 0.2-0.5 percent levels it was bactericidal (Rasic and Kurmann, 1983). Therefore, the objective of the present study was to study the relationship between the ability to tolerate bile acids and deconjugation activity of bifidobacteria.

Materials and Methods

Source, preservation and recovery of cultures: Cultures of *Bifidobacterium bifidum* (ATCC 35914), *B. adolescentis* (ATCC 11146), *B. breve* (ATCC 15698), *B. breve* (ATCC 1 5701), *B. infantis* (ATCC 279201 and *B. longum* (ATCC 15708) were obtained from the American Type Culture Collection (ATCC). The cultures were preserved in STC Bead Storage System (Technical Services Consultants Limited, UK).

They were recovered in TPY broth (Scardovi, 1986), incubated anaerobically at 37°C for 48 hours (BBL Gaspak Anaerobic System). Sub culturing was done by using 1 percent inoculum into TPY broth and incubated anaerobically for 18 hours at 37°C.

Comparison of cultures for bile tolerance: Method from Walker and Gilliland (1993) was used to compare the ability of bifidobacteria to grow in the presence of 0.3 percent oxgall. The cultures were incubated, anaerobically for 3 hours, at 37°C. The increase in absorbance was measured at 620 nm were used to compare growth of the cultures. All bile media were sterilised by autoclaving at 121°C for 15 minutes.

Screening cultures for deconjugation of sodium taurocholate: Method from Walker arid Gilliland (1993) was used to measure the amount of free cholic acid liberated by the culture. Twenty ml of sterilised TPY broth containing

0.05 percent sodium taurocholate of each culture was adjusted to pH 7.0 with 1 N NaOH. The cells were removed by centrifugation for 10 minutes at 12,000 x g at 4°C using a BECKMAN J2-21 M/E centrifuge (with rotor JA-20). Fifteen millilitres of the resulting supernatant fluid were adjusted to pH 1.0 using 10 N HCI and increased to 24 ml with addition of distilled water. Three millilitre. portions of each sample were transferred to glass stoppered test tubes to which 9 ml volumes of ethyl acetate were added. The contents of each tube were vortexed and the phases were allowed to separate. Three millilitre of ethyl acetate layer from each tube were transferred to a clean test tube and evaporated to dryness at 60°C under flow of nitrogen gas. One millilitre of 0.01 N NaOH was added to each tube to dissolve the residue. Six millilitres of 16 N H_2SO_4 were then added to each tube, followed by the addition of 1 ml furfuraldehyde: The solution were mixed, heated for 13 minutes in a 65°C water bath, and cooled to room temperature. Five millilitres of glacial acetic acid were added to each tube and mixed, after which the absorbance at 620 nm was measured against a reagent blank and compared with a standard curve to determine the concentration of free cholic acid.

Statistical analysis: Data were analysed using the ANOVA procedure from SAS. Duncan's Multiple Range Test was used to determine differences occurred among the means.

Results

Comparison of cultures for bile tolerance: The ability to grow in the presence of bile varied among cultures tested as shown in Table 1. *Bifidobacterium longum* (ATCC 15708) grew significantly ($p<0.05$) better than other cultures. *Bifidobacterium breve* (ATCC 15698) was the most sensitive to bile, exhibiting significantly ($p<0.05$) less growth than all other cultures except *B. infantis* (ATCC 27920).

Table 1: Comparison of bile tolerance of *Bifidobacterium* sp.

Culture	Changes in absorbance[2]
B. longum (ATCC 15708)	0.066[a]
B. adelescentis (ATCC 11146)	0. 05[b]
B. bifidum (ATCC 35914)	0.039[c]
B. breve (ATCC 15701)	0.021[d]
B. infantis (ATCC 27920)	0.016[ed]
B. breve (ATTCC 15698)	0.006[e]

[a-e]Means with superscript letters do not differ significantly ($p\geq0.05$)

[1]based on growth in TPY broth supplemented with 0.3% oxgall. Each value is a mean of two trial

[2]Absorbance read at 620 nm and measured after 3 hours of incubation

Screening of cultures for deconjugation of sodium taurocholate: Result from screening cultures for deconjugation of sodium taurocholate is shown in Table 2. Data obtained were the amount of cholic acid liberated from deconjugation activity. The higher the concentration showed the higher deconjugation activity has occurred. Variations among strains in the ability to deconjugate sodium taurocholate were significant (p 0.05).

Deconjugation activity of all strains tested were significantl ($p<0.05$) different from control. For the first 4 hours incubation time, *B. bifidum* (ATCC 35914) was able deconjugate sodium taurocholate thus releasing cholic was significantly higher ($p<0.05$) than other culture After 4 hours of incubation time up to 8 hours, rapi deconjugation activity occurred for *B. infantis* (ATCC 27920), *B. bifidum* (ATCC 35914) and *B. breve* (ATCC 15698), where *B. infantis* (ATCC 27920) show significantly ($p<0.05$) highest concentration (0.001 μmol/ml) of released cholic acid. No significant different ($p<0.05$) observed for *B. adolescentis* (ATCC 11146), longum (ATCC 15708) and *B. breve* (ATCC 15701).

Maximum deconjugation of sodium taurocholate w observed at 18 hours of incubation time for *B. infan* (ATCC 27920), *B. breve* (ATCC 15698) and *B. bifidu* (ATCC 35914). *B. infantis* (ATCC27920) released: significantly (p 0.05) the highest concentration (0.01 μmol/ml) of cholic acid followed by *B. breve* (ATCC 1569 (0.013 μmol/ml) and *B. bifidum* (ATCC 35914) (0.00 μmol/ml). Although *B. bifidum* (ATCC 35914) released cholic acid faster and higher for the first 5 hours incubation time than other cultures, the final amount of f cholic acid liberated was lower than *B. infantis* (ATCC 27920) and *B. breve* (ATCC 15698). No significant difference ($p<0.05$) was observed between *B. long* (ATCC 15708) and *B. adolescentis* (ATCC 11146) , between *B. longum* (ATCC 15708) and *B. breve* (ATCC 15701). But the difference was significant ($p<0.05$) *B. adolescentis* (ATCC 11146) and *B. breve* (ATCC 1570). All cultures tested showed significant difference (p 0.05) in cholic acid concentration for 24 hours incubation time. Highest concentration (0.0151 μmol/ml) was observed *B. infantis* (ATCC 27920) and lowest concentrat (0.0009 μmol/ml) was observed for *B. breve* (ATCC 15701).

Bifidobacterium infantis (ATCC 27920) showed significant difference ($p<0.05$) in released cholic acid as time increased from 0 to 18 hours, but the amount was not significant ($p<0.05$) from 18 to 24 hours incubation time. Sim trends were observed for *B. bifidum* (ATCC 35914). For *B. breve* (ATCC 15698), the increased concentration cholic acid was not significant ($p<0.05$) from 0 hours incubation time. But significant ($p<0.05$) increase was observed after 4 hours up to 18 hours. 18 hours incubation time, the increased concentration cholic acid released was not significantly ($p<0.05$) Increased concentration of released cholic acid was significant from 0 to 4 hours incubation time for *adolescentis* (ATCC 11146). For 8 hours, the increased concentration measured was not significantly different ($p<0.05$) from the concentration of cholic acid released

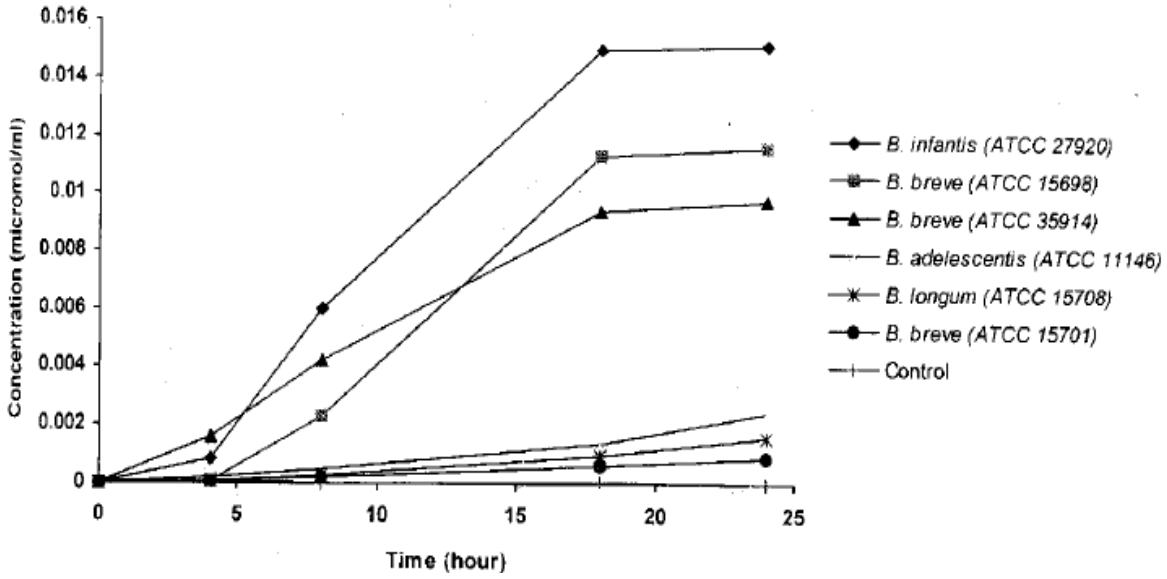

Fig. 1: Relationship between concentration of released cholic acid (μmol/ml) and incubation time (hour)

hours incubation time. But this amount was significantly different ($p \leq 0.05$) from 0 hour incubation time. After 8 hours up to 24 hours the increased concentration was significantly different ($p \leq 0.05$). This observation was similar to *B. breve* (ATCC 15701).

For *B. longum* (ATCC 15708), the increased in concentration of cholic acid released was not significantly different ($p \leq 0.05$) from 0 to 8 hours incubation time. However after 8 hours, the cholic acid measured increased significantly higher at ($p \leq 0.05$). To get a better view of the result obtained, a graph that shows a relationship between concentration of cholic acid released and time of incubation for each *Bifidobacterium* spp was drawn in Fig. 1.

Discussion

All cultures of bifidobacteria tested exhibited various degree of bile tolerance. Degree of bile tolerance of the culture is an important characteristic that enables culture to survive in the upper parts of intestinal tract.

Deconjugation of sodium taurocholate will liberate cholic acid, This phenomenon resulted from hydrolysis reaction of the amide bond of sodium taurocholate by hydrolase enzyme, which is localised at the membrane wall of the bacteria.

For all cultures, increasing of cholic acid concentration were noted significantly ($p \leq 0.05$) after 4 hours of Incubation time, except for B. bifidum (ATCC 35914) which released cholic acid faster than other culture tested. Perhaps this is due to lag phase (Gilliland *et al*., 1984). The deconjugation started only when bacteria began to grow (4 hours), afterwards the deconjugation rate increased linearly between 4 and 18 hours. After 18 hours, deconjugation continued until complete biotransformation of taurocholic but the increasing were not significant ($p \leq 0.05$) for *B. infantis* (ATCC 27920), *B. breve* (ATCC 15698) and *B. bifidum* (ATCC 35914). Possibly this corresponds to the beginning of the stationary phase growth. Gilliland and Speck (1977) reported that, in this phase the low pH that resulted from acid produced by the culture likely inhibited enzyme activity. The optimum pH for the bile salt deconjugation by lactobacilli was approximately 6.0.

Bifidobacterium infantis (ATCC 27920) and *B. breve* (ATCC 15698) which were the least bile tolerant, showed considerable ability to deconjugate sodium taurocholate. Maybe, some of the difference in bile tolerance was due to the natural difference in growth of the individual culture. Gilliland *et al*. (1984) reported that strains *Lactobacillus acidophilus* that is less bile tolerant tended to have a longer lag time. This trait when coupled with expression of very active deconjugation systems would be more subject to inhibition caused by the released of more free cholic acid prior to initiation growth (Walker and Gilliland, 1993). Floch *et al*. (1972) reported that deconjugated bile acids were inhibitorier to microorganisms than conjugated bile acids and suggested that this inhibitory effect may play a role in inhibiting microbial growth in the intestinal tract.

Bifidobacterium adelescentis (ATCC 11146) and *B. longum* (ATCC 15708) which were good bile tolerant showed a less deconjugation activity of sodium taurocholate to release cholic acid. Gilliland and Speck (1977) reported that ability to deconjugate bile salts varied among lactobacilli isolated from human faeces. Some failed to deconjugate either

Table 2: Concentration of cholic acid liberated by deconjugation activity of *Bifidobacterium* sp. during 24 hours incubatii at 37°C (μmol/ml)

Culture	Time				
	0	4	8	18	24
B. infantis (ATCC 27920)	0.000^{Da}	0.0008^{Cb}	0.0060^{Ba}	0.0150^{Aa}	0.0151
B. breve (ATCC 15698)	0.000^{Ca}	0.0001^{Cc}	0.0023^{Bc}	0.0113^{Ab}	0.011^{E}
B. bifidum (ATCC 35914)	0.000^{Da}	0.0016^{Cc}	0.0042^{Bb}	0.0094^{Ac}	0.009^{i}
B. adolescentis (11146)	0.000^{Da}	0.0002^{Cc}	0.0005^{cd}	0.0014^{Bd}	0.0024
B. longum (ATCC 15708)	0.000^{Ca}	0.0001^{Cc}	0.0003^{Ced}	0.0010^{Bed}	0.0016^{E}
B. breve (ATCC 15701)	0.000^{Da}	0.0001^{CDc}	0.0002^{Cad}	0.0005^{Bef}	0.0009
Control	0.000^{Aa}	0.0001^{Ac}	0.0000^{Ae}	0.0001^{Af}	0.0000

$^{A,B,\ C,D}$Means with the same letter within rows are not significantly different ($p \geq 0.005$)

^{a,b,c,d,e,f,g}Means with the same letter within columns are not significantly different ($p \geq 0.005$) [1]Unit in hours.

taurocholate or glycocholate, some deconjugate only one of the two, and some were active in both. This can explained why *B. adolescentis* (ATCC 11146) and *B. longum* (ATCC 15708) that are good bile tolerant showed less deconjugation activity. Walker and Gilliland (1993) concluded that correlation among bile tolerance, deconjugation activity and assimilation of cholesterol was not significant.

The action of bifidobacteria species on conjugated bile salt, where percentage and initial rate of deconjugation were determined. They used dihydroxyconjugated bile salt (i.e., tauro-and glycochenodeoxycholic acid, tauro and glycodeoxycholic acid) and trihydroxyconjugated bile salt (i.e., tauro and glycocholic acid) at 4 mM concentration. Percentage of deconjugation was determined after 30 minutes of incubation time at 37°C. They observed that glycoconjugated bile salt were generally better hydrolysed than trihydroxyconjugated salts. Great differences were observed with taurocholic acid. All strains tested were deconjugated this bile salt weakly and slowly except for *B. longum* (ATCC 15708). Result obtained from this studies can not be compared to the result from their studies because the method used was not the same. Clark and Martin (1994) reported that *Bifidobacterium longum* (ATCC 15708) exhibited best tolerance to bile by surviving at both 2 and 4 percent levels after 12 hours, whereas *B. infantis* (ATCC 27920), *B. adolescentis* (ATCC 11146) and *B. bifidum* (ATCC 35914) did not survive in 2 percent oxgall after 12 hours incubation. Their result was the same as result from our study as *B. longum* (ATCC 15708) was the best tolerance to bile. There are limiting factors in comparing their studies with our studies. They used higher concentration of oxgall and they did not use growth support media or buffer during exposure to bile concentration.

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