Strain Differences in Deconjugation of Bile Acids in *Bifidobacterium pseudocatenulatum* Isolates

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The survival and growth rate of twenty eight isolates of bifidobacteria in bile were evaluated. Of 28 isolates, 25 were tolerance towards 2.0% concentration of bile while 14 isolates were tolerance towards 4.0% of bile after 12 hours of exposure. Six isolates of bifidobacteria with higher tolerance to 4.0% of bile were further evaluated for their ability to deconjugate different types of bile acids namely taurocholic acid (TC), glycocholic acid (GC), taurochenodeoxycholic acid (TCDC), glycochenodeoxycholic acid (GCDC), taurodeoxycholic acid (TDC), and glycodeoxycholic acid (GDC). Three *Bifidobacterium pseudocatenulatum* isolates (D22, F117, and G4) were found to have the similar deconjugation activity and were able to deconjugate 78.6–84.6% (TC), 98.9–99.9% (GC), 87.9–97.5% (TCDC), 91.1–100.0% (GCDC), 83.7–87.8% (TDC) and 96.5–99.0% (GDC).

Key words: *Bifidobacterium* spp.; probiotic; deconjugation; bile salt hydrolase

INTRODUCTION

Mammalian bile acids are C₂₄ steroid carboxylic acid molecules, which are synthesized in the liver as free bile acids and then conjugated to either glycine or taurine via an amide bond (22). These conjugated bile acids are then released into the small intestine where they act primarily as emulsifiers of dietary lipids (27). Most conjugated bile salts excreted (about 97%) are reabsorbed from the small intestine and returned to the liver through the hepatic-portal circulation (26). Several genera of intestinal bacteria including *Enterococcus*, *Peptostreptococcus*, *Bifidobacterium*, *Fusobacterium*, *Clostridium*, *Bacteroides*, and *Lactobacillus* (3, 7, 23) produce bile salts hydrolases (BSH) which cleave the amino acid moieties from conjugated bile acids. The majority of bacteria capable of deconjugation are strictly anaerobes. They can biotransform bile acids into a number of different metabolites. Normal human faeces may contain more than 20 different bile acids that have been formed from the primary bile acids, cholic acid and chenodeoxycholic acid (1, 13, 29, 32). The resulting free bile acids from deconjugation process are less efficiently recycled by the enterohepatic circulation (8).

Deconjugation or dehydrogenation of 3 α -, 7 α - and 12 α -OH groups and 7 α -dehydroxylation are the prin-

cipal and important types of biochemical reactions brought about by intestinal microflora on bile acids (19). It has been demonstrated that *Bifidobacterium* spp. are capable of splitting the amide bond of taurocholic and glycocholic acids (6, 12, 21, 30).

Ferrari et al. (14) further confirmed the ability of bifidobacteria strain to deconjugate taurocholic and glycocholic acid when they found 48 out of 52 isolates of *Bifidobacterium* spp. studied acted on both the conjugated bile acids. They concluded that the ability to deconjugate taurocholic and glycocholic acids is widespread among members of *Bifidobacterium*. Thus, it may be assumed that bifidobacteria, one of the main bacterial groups in the gut, have an important role in the transformation of bile acids in the intestine. In this work, we studied the deconjugation of bile salts by difference strains of *Bifidobacterium pseudocatenulatum* in the bile salt-TPY medium.

MATERIALS AND METHODS

Bacteria and chemicals. Sodium salts of taurocholic (TC), glycocholic (GC), taurodeoxycholic (TDC), glycodeoxycholic (GDC), taurochenodeoxycholic (TCDC), glycochenodeoxycholic (GCDC) were obtained from Sigma (Sigma Chemical Co., USA). A bile solution was prepared using 10 g (w/v) of powdered oxgall (Lot no. 106726JA) (Difco Laboratories, Detroit, USA) in 90 ml distilled water. The *Bifidobacterium pseudocatenulatum* isolates used in this study, were from stock culture collection of probiotic laboratory at

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Universiti Putra Malaysia. In addition, *Bifidobacterium pseudocatenulatum* was the only species of bifidobacteria that could be isolated from faecal samples of Malaysian infants. All the isolates were identified by biochemical reaction and 16S rDNA sequences (31). All the 28 bifidobacteria isolates were cultured in Trypticase Phytone Yeast extract (TPY) medium, as recommended by Scardovi (28).

Growth of bifidobacteria in the presence of bile. The bile tolerance of the bifidobacteria was determined by the method adapted and modified from Gilliland et al. (17) and Clark and Martin (9). Ten milliliter of TPY broth with 2.0% or 4.0% (v/v) of bile solution were placed in test tubes and sterilized at 121°C for 15 min. TPY broth without bile solution was used as a control. All the test tubes were then inoculated with 1% of freshly grown cultures of each bifidobacteria strain to be tested. The cultures were then incubated at 37°C, anaerobically. Growth was monitored by measuring the absorbance at 620 nm (A_{620nm}) using a spectrophotometer at time intervals of 0, 3, 6, 9, and 12 hours. The experiment was conducted in duplicate.

Determination of hydrolase activity. Six isolates of bifidobacteria with highest tolerance to bile were further evaluated for their hydrolase activity. Test broths were prepared by adding either 5 mm or 10 mm of different bile solutions to 10 ml TPY broth and sterilized at 121°C for 15 min. All the tubes were inoculated with 1% of an overnight culture and incubated anaerobically at 37°C for 24 hours. Conjugated bile salts were identified and quantified using high pressure liquid chromatography with some modifications as described by Tietz et al. (33). The amount of each bile salt deconjugated was based on the disappearance of each conjugated bile salt from the growth medium and expressed as percentage of deconjugation activity.

Statistical analysis. A 3×28 factorial design was used in the earlier experiment with bile salt (3 concentrations) and bifidobacteria (28 isolates) to determine statistical significance ($p < 0.05$). All the data were analyzed using analysis of variance (ANOVA) of the SAS (1990). A $6 \times 2 \times 8$ factorial design was further used in the experiment of bile salt deconjugation activity (6 treatments and 2 concentrations) and bifidobacteria (8 isolates). Coefficients of variation (CV) analyses were used to compare treatment average for which the analysis of variance indicated significant ($p < 0.05$) difference.

RESULTS

In the first part of the study, 28 isolates of bifidobac-

teria were tested for survival in 0.0%, 2.0% (maximum concentration normally found in the human small intestine), and 4.0% (v/v) (twice the concentration found in the human small intestine) as reported by Davenport (11). The ability of the bifidobacteria isolates to grow in the TPY broth with 2.0 and 4.0% (v/v) and without oxgall revealed substantial diversity among isolates (Table 1). The oxgall was found to exert inhibitory effect on all isolates as observed by decrease in absorbance in 2.0% and 4.0% (v/v) oxgall as compared to the control. The time required for all the isolates to increase the initial absorbance by 0.3 units was considerably higher in TPY broth with 2.0 and 4.0% (v/v) oxgall than the control (TPY broth without oxgall) (Table 2). *B. infantis* F112 was the most resistant isolate in 4.0% concentration. The difference in time required to increase the initial absorbance by 0.3 unit was 23.67%, being the lowest among all the culture tested. *B. infantis* F117 had the second lowest time difference (35.71%), followed by *B. breve* D22 (40.06%), *B. infantis* Z45 (45.38%), *B. infantis* G4 (52.17%) and *B. breve* G48 (84.03%). *B. infantis* Z45 ranked fourth according to tolerance to 4.0% concentration of bile, although it had the fastest growth rate in TPY broth compared to the other five isolates. Only 3 out of 28 isolates, *B. infantis* C15, *B. infantis* D36, and *B. breve* F64 did not show any increase in turbidity in the presence of 2.0% of oxgall throughout the 12 hr incubation time, indicating their inability to grow in the presence of bile. Apparently 4.0% concentration of oxgall had more inhibitory effect on the growth rate as 14 out of 28 isolates did not show any increase in turbidity during 12 hr exposure time (Table 1).

In the second part of the study, six isolates of bifidobacteria with highest tolerance to bile were subjected to 5 mm and 10 mm of different types of bile acids. All the six isolates tested by HPLC for the deconjugation of TC, GC, TCDC, GCDC, TDC and GDC acted on both tauro- and glyco-conjugated bile acids, and the percentages of deconjugation and coefficient of variation obtained are given in Tables 3 and 4. At 5 mm of bile salts, significant differences in deconjugation activity were observed for *B. infantis* D22 in TC, *B. infantis* F117 in GC and in TCDC, *B. infantis* G4 in GCDC and in GDC and *B. infantis* G17 in TDC (Table 3). At 10 mm of bile salts, significant differences in deconjugation activity of GC, GCDC, TDC and GDC were obtained with isolate *B. infantis* G4. *B. infantis* F117 and *B. infantis* D22 showed significant deconjugation of TC and TCDC, respectively (Table 4). Overall the percentage of the deconjugation activity was

Table 1. Survival of *Bifidobacterium* spp. in the absence (control) and presence of 2.0% and 4.0% of oxgall¹ after 12 hr exposure time.

Test organisms ²	Absorbance (A _{620 nm})		
	Control	2.0% of oxgall	4.0% of oxgall
<i>B. breve</i> A50	1.005	0.406	—
<i>B. breve</i> C11	0.966	0.347	—
<i>B. infantis</i> C15	0.885	—	—
<i>B. infantis</i> C17	0.689	0.612	0.539
<i>B. breve</i> C20	0.994	0.471	—
<i>B. infantis</i> C25	0.905	0.339	—
<i>B. infantis</i> C83	0.998	0.346	—
<i>B. breve</i> D19	0.845	0.495	0.375
<i>B. infantis</i> D22	1.146	0.637	0.552
<i>B. breve</i> D73	0.911	0.481	0.485
<i>B. infantis</i> D36	0.895	—	—
<i>B. infantis</i> F117	0.833	0.578	0.558
<i>B. breve</i> F58	0.862	0.492	0.464
<i>B. infantis</i> F112	0.903	0.619	0.572
<i>B. breve</i> F100	1.026	0.374	—
<i>B. infantis</i> F69	0.891	0.563	0.398
<i>B. infantis</i> F34	0.780	0.402	0.325
<i>B. infantis</i> F91	1.176	0.489	—
<i>B. infantis</i> F66	0.770	0.584	0.423
<i>B. breve</i> F64	1.067	—	—
<i>B. breve</i> F81	0.798	0.589	—
<i>B. infantis</i> G4	0.790	0.612	0.504
<i>B. breve</i> G48	1.029	0.589	0.389
<i>B. infantis</i> G41	1.032	0.362	—
<i>B. infantis</i> Z80	0.971	0.387	—
<i>B. infantis</i> Z45	0.916	0.658	0.586
<i>B. infantis</i> Z47	0.926	0.439	—
<i>B. infantis</i> Z37	0.875	0.574	0.483

¹ A dehydrated and purified fresh bile obtained from Difco Laboratories, Detroit, USA.

² All the isolates used in this experiment were obtained from the stock culture collection of the probiotic laboratory, Universiti Putra Malaysia (Yazid et al., 1999).

higher in TPY broth supplemented with 5 mM bile acids ($p < 0.05$) compared to the TPY broth plus 10 mM of bile acids.

DISCUSSION

Gastrointestinal systems have varying concentrations of bile. The rate of secretion of bile acids and its concentration depend on the type of food consumed. For example, the fat content of the diet could influence the level of fecal bile acids and fatty acids may increase the inhibitory effects of bile acids towards *Lactobacillus* and *Bifidobacterium* spp. (2). After a meal, bile salt concentration sharply increases in the duodenum up to approximately 15 mM and then progressively decreases to 5 mM (20).

Bifidobacteria were reported to be able to survive physiological and higher bile salt levels (24). At 0.06 to 0.03% sodium glycocholate, *B. infantis* showed the best survival followed by *B. bifidum* and *B. breve*, whereas *B. longum* showed the least resistance to bile. Our present results are similar where *B. infantis* showed better resistance to 2.0 and 4.0% concentration of bile as compared to *B. breve*, although the concentration of bile used in their study was less than in our experiment. However, in contrary, study conducted by Clark and Martin (9) revealed that *B. infantis*, *B. adolescentis*, and *B. bifidum* did not survive at the 2.0% and 4.0% level of bile acids after 12 hr incubation time, however, viable cells of *B. longum* in 4.0% oxgall were still surviving at a rate of 10^7 cfu/ml. A limiting factor in com-

Table 2. Comparison of growth¹ rate of *Bifidobacterium* spp. in TPY broth without and with 2.0% and 4.0% of oxgall.²

Rank order ⁴	Isolates	Hour ³			% Increase in time ⁵	
		TPY broth	TPY broth + 2.0% oxgall	TPY broth + 4.0% oxgall	2.0% oxgall	4.0% oxgall
1	F112	2.83 ± 0.49	3.29 ± 0.44	3.50 ± 0.38	16.25	23.67
2	F117	2.80 ± 0.32	3.74 ± 1.06	3.80 ± 0.27	33.57	35.71
3	D22	2.97 ± 0.33	3.40 ± 0.47	4.16 ± 1.08	14.47	40.06
4	Z45	2.6 ± 0.10	3.30 ± 0.41	3.78 ± 1.95	26.92	45.38
5	G4	2.76 ± 0.17	3.78 ± 0.64	4.20 ± 0.71	36.95	52.17
6	G48	3.82 ± 0.78	4.12 ± 1.13	7.03 ± 0.51	7.85	84.03
7	F58	3.9 ± 0.48	7.29 ± 1.20	8.85 ± 1.53	86.92	126.92
8	Z37	1.80 ± 0.74	3.32 ± 0.72	4.34 ± 1.15	84.44	141.11
9	F69	2.45 ± 1.21	3.27 ± 0.73	7.22 ± 1.37	33.46	194.69
10	F66	2.82 ± 0.50	3.61 ± 0.20	8.51 ± 0.14	28.01	201.77
11	C17	2.02 ± 0.52	3.76 ± 0.29	6.30 ± 0.43	86.13	211.88
12	D73	1.62 ± 1.08	4.46 ± 0.46	5.12 ± 0.84	97.24	216.04
13	D19	2.29 ± 1.12	4.98 ± 0.24	7.39 ± 1.06	117.46	222.70
14	F34	2.57 ± 0.93	5.62 ± 0.39	8.68 ± 0.09	118.67	237.74
15	A50	4.13 ± 1.20	7.50 ± 0.71	a	81.59	—
15	C11	3.67 ± 0.69	7.80 ± 0.72	a	112.53	—
15	C15	4.48 ± 0.69	a	a	—	—
15	C20	4.66 ± 0.90	6.73 ± 1.03	a	44.42	—
15	C25	3.78 ± 1.00	10.6 ± 0.77	a	180.42	—
15	D36	2.11 ± 0.32	a	a	—	—
15	F100	5.15 ± 0.96	8.46 ± 1.87	a	64.27	—
15	F91	2.88 ± 0.75	5.76 ± 0.57	a	100.00	—
15	F64	5.05 ± 0.93	a	a	—	—
15	F81	2.29 ± 0.72	3.26 ± 0.45	a	42.35	—
15	G41	4.39 ± 0.53	8.23 ± 1.07	a	87.47	—
15	Z80	5.17 ± 1.38	7.47 ± 1.09	a	44.48	—
15	Z47	3.42 ± 0.65	6.53 ± 1.03	a	90.93	—

¹ Incubated anaerobically (BBL GasPak System, Becton Dickinson, USA) at 37°C for 12 hours.² A dehydrated and purified fresh bile obtained from Difco Laboratories, Detroit, USA.³ Mean ± SD hours required for absorbance to increase 0.3 unit. Each mean is an average of four determination.⁴ Ranked from shortest to longest time required for growth in broth with 4.0% oxgall.⁵ Percent increase in time needed for the absorbance ($A_{620\text{ nm}}$) to increase by 0.3 units in TPY broth with 2.0 and 4.0% oxgall.

a: No growth was observed.

paring Clark and Martin (9) study and others is that they did not use any growth support media or buffer subjected to bile concentration while in our case and others, some type of growth media with bile salts incorporated into them were used. Strain-dependency of bifidobacteria and lactobacilli to survive under different concentrations of bile was observed by Lankaputhra and Shah (25). They pointed out that while one strain of *B. longum* (1941) was tolerant to bile, the other strain of *B. longum* (20097) did not survive well in bile.

Generally, all the tested *Bifidobacterium* isolates in the present experiment deconjugated glycoconjugates better than tauroconjugated bile salts significantly ($p <$

0.05) and showed substrate specificity. This is in agreement with Brashears et al. (5) and Corzo and Gilliland (10). Study of the substrate specificity of bile salt hydrolase from human faecal cultures (4) has demonstrated that the portion of the amide bond and the introduction of various amino acids at or around the amide bond influence the rate of hydrolysis. Gopal-Srivastava and Hylemon (18) have demonstrated that glycine and taurine conjugates of cholic acid and deoxycholic acids are hydrolyzed; however, taurine conjugates were hydrolyzed less readily than glycine conjugates.

As revealed in this study, *B. infantis* F112 which has the fastest growth in the 4.0% oxgall (Table 2), was not

Table 3. Percentage of deconjugation obtained with selected bifidobacteria isolates on different bile acids (5 mM).

Strain	Biliary bile					
	TC	GC	TCDC	GCDC	TDC	GDC
C17	77.7 ^a (7.3)	87.7 ^{ab} (2.4)	59.0 ^b (6.0)	72.3 ^c (3.9)	89.2 ^a (5.5)	69.8 ^b (1.0)
D22	84.6 ^a (3.3)	99.2 ^a (0.7)	87.9 ^a (4.0)	91.1 ^a (2.3)	83.7 ^a (1.7)	97.5 ^a (2.2)
F112	59.1 ^b (6.0)	82.8 ^b (5.1)	58.7 ^b (7.2)	92.7 ^a (4.6)	65.1 ^b (5.4)	96.6 ^a (2.2)
F117	78.6 ^a (3.6)	99.9 ^a (0.0)	97.5 ^a (2.2)	96.2 ^a (2.9)	84.3 ^a (3.3)	96.5 ^a (1.5)
G4	83.0 ^a (4.2)	98.9 ^a (0.7)	93.0 ^a (5.3)	100.0 ^a (0.0)	87.8 ^a (0.0)	99.0 ^a (0.7)
Z45	69.9 ^{ab} (1.0)	98.8 ^a (0.7)	93.4 ^a (5.3)	98.2 ^a (1.4)	86.0 ^a (4.9)	94.9 ^a (3.7)

The same cellular concentration was used and the percentage of deconjugation was determined after 24 hr at 37°C. Each value represents the average from two trials; values in the parentheses represent the coefficient of variations. Values with the same letter are not significantly different.

Abbreviations: TC, taurocholic acid; GC, glycocholic acid; TCDC, taurochenodeoxycholic acid; GCDC, glycochenodeoxycholic acid; TDC, taurodeoxycholic acid; GDC, glycodeoxycholic acid.

Table 4. Percentage of deconjugation obtained with selected bifidobacteria isolates on different bile acids (10 mM).

Strain	Biliary bile					
	TC	GC	TCDC	GCDC	TDC	GDC
C17	64.6 ^{ab} (7.6)	63.6 ^b (5.6)	60.0 ^{bc} (5.9)	65.3 ^c (5.4)	46.3 ^b (7.1)	68.7 ^c (6.2)
D22	73.2 ^{ab} (8.7)	95.5 ^a (3.7)	84.7 ^a (0.0)	89.5 ^{ab} (0.8)	82.2 ^a (6.0)	89.1 ^{ab} (4.0)
F112	58.5 ^{ab} (11.2)	74.9 ^b (6.6)	47.9 ^c (11.8)	88.4 ^{ab} (3.2)	42.8 ^b (1.6)	91.1 ^{ab} (3.9)
F117	78.0 ^a (7.2)	92.3 ^a (4.6)	62.8 ^b (2.2)	92.7 ^{ab} (4.6)	83.0 ^a (1.7)	95.8 ^{ab} (3.0)
G4	74.8 ^{ab} (6.6)	99.0 ^a (0.7)	75.5 ^{ab} (6.5)	98.8 ^a (0.7)	84.0 ^a (0.0)	98.1 ^a (1.4)
Z45	53.2 ^b (0.0)	96.9 ^a (0.7)	70.2 ^{ab} (9.0)	94.1 ^{ab} (4.5)	83.8 ^a (1.7)	92.3 ^{ab} (3.0)

The same cellular concentration was used and the percentage of deconjugation was determined after 24 hr at 37°C. Each value represents the average from two trials; values in the parentheses represent the coefficient of variations. Values with the same letter are not significantly different.

Abbreviations: TC, taurocholic acid; GC, glycocholic acid; TCDC, taurochenodeoxycholic acid; GCDC, glycochenodeoxycholic acid; TDC, taurodeoxycholic acid; GDC, glycodeoxycholic acid.

the most active one towards deconjugation activity. This could be due to the fact that deconjugated bile acids were more inhibitory to microorganisms than conjugated bile acids and suggested that this inhibitory ef-

fect may play a role in inhibiting microbial growth in the intestinal tract (15, 16). *Lactobacillus acidophilus* ATCC 4356, which was the least bile-resistant strain tested, deconjugated approximately twice as much so-

dium taurocholate as did strains ATCC 43121 or NCFM-L during 3 hr of incubation. Therefore, the increased deconjugation may explain why this strain is less bile-resistant than the other two strains.

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