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Acid and bile tolerance of *Lactobacillus* isolated from chicken intestine

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L.Z. JIN, Y.W. HO, N. ABDULLAH AND S. JALALUDIN. 1998. Twelve *Lactobacillus* strains isolated from chicken intestine were used to investigate acid and bile tolerance *in vitro*. Ten out of the 12 strains were slightly affected by 0·3% bile salts, showing a delay of growth (d) of 0·6–37·2 min compared with growth in control cultures. Two strains were not affected by the bile salts. Of the 12 strains, seven could be arbitrarily classified as resistant (d < 15 min) and five as tolerant (15 min < d ⩽ 40 min). *Lactobacillus* strains from the caecum showed better tolerance to acid than those from the ileum. Generally, the survival of the ileal strains was very low at pH 1·0 and 2·0, and moderate at pH 3·0. In contrast, caecal *Lactobacillus* strains could survive at pH 1·0 for up to 2 h of incubation; growth was moderate at pH 2·0 and good at pH 3·0 and 4·0.

INTRODUCTION

The effects of probiotics (direct-fed microbials) on animal performance have been well studied, but the results are often contradictory (Jin *et al*. 1997). Some studies have shown that probiotics enhance the growth and performance of animals (Fuller 1989) while others have indicated that there are no beneficial effects on animals (Watkins and Kratzer 1983, 1984). The variations in results may be due to differences in the species or strains of micro-organisms used, or to the variations in concentration of viable microbes supplemented in the diet. Recently, emphasis has been placed on the selection and preparation of *Lactobacillus* strains as probiotics (Havenaar *et al*. 1992; Chateau *et al*. 1994). Other factors, such as tolerance to bile and low gastrointestinal pH, also need to be considered if the desired results are to be obtained from the use of lactobacilli as growth promotants.

Most bacteria do not survive well at low pH values. The severe acidic conditions of the crop, proventriculus and gizzard could have an adverse effect on the bacteria. Thus, it has been suggested that microbial cultures to be used as growth promotants should be screened for their resistance to acidity (Conway *et al*. 1987).

Once the bacteria reach the intestinal tract, their ability to survive depends on their resistance to bile (Gilliland *et al*. 1984; Gilliland 1987). Bile entering the duodenal section of the small intestine has been found to reduce survival of bacteria. This is probably due to the fact that all bacteria have cell membranes consisting of lipids and fatty acids which are very susceptible to destruction by bile salts. Hence, the success of a probiotic also depends on the selected strain possessing bile-resistant qualities.

The objective of this study was to investigate the acid and bile tolerance of moderately or strongly adherent *Lactobacillus* strains isolated from chicken intestine.

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MATERIALS AND METHODS

Lactobacillus cultures

Twelve *Lactobacillus* strains (six strains of *Lact. brevis*, three of *Lact. fermentum*, two of *Lact. acidophilus* and one of *Lact. crispatus*), which were isolated from washed sections of gut tissues excised from 3-week-old broilers, were used. These *Lactobacillus* strains, which showed moderate or strong ability to adhere to ileal epithelial cells, were the same as those identified (using the API kit system) and described by Jin *et al*. (1996). Stock cultures of the *Lactobacillus* strains were stored in vials (Protect, Heywood, UK) at −70 °C in a freezer. All the strains were cultured anaerobically in MRS broth (Oxoid) at 37 °C for 18 h or overnight.

Bile tolerance test

The method used for testing bile tolerance was similar to that described by Gilliland *et al.* (1984). The *Lactobacillus* strains were grown overnight in MRS broth and 0·1 ml of the culture suspension was inoculated into tubes containing 10 ml of MRS broth with 0·3% chicken bile (Sigma) or without bile (which acted as controls). The inoculated tubes were incubated at 37 °C. Three tests, each with a duplicate, were carried out on each *Lactobacillus* strain in each treatment. Growth was monitored hourly for 6 h by increased absorbance at λ 660 nm using a spectrophotometer (DU-65, Bechman, Fullerton, USA). Growth curves were plotted and the times required for turbidity to obtain an absorbance of 0·3 were determined for both the control cultures and the cultures with bile. The difference between the control and bile culture (d), expressed in minutes, was considered as delay in growth as a result of inhibition by bile (Chateau *et al.* 1994).

Acid tolerance test

The method of Conway *et al.* (1987) was employed to study the acid tolerance of the 12 *Lactobacillus* strains. The cultures were grown in MRS broth (Oxoid) at 37 °C overnight, then subcultured into 10 ml of fresh MRS broth and incubated for another 24 h. The cultures were centrifuged at 2000 *g* for 10 min at 4 °C, the pellets washed twice in sterile phosphate-buffered saline (PBS, pH 7·2; Sigma) and resuspended in 1 ml of PBS. For each *Lactobacillus* strain, 0·1 ml of culture suspension was added separately into a series of tubes containing 2 ml of sterile PBS at various pH values. For *Lact. acidophilus* I 26 and *Lact. fermentum* I 25, the pH values tested were 0·5, 1, 2, 3, 4 and 5, and for the rest of the *Lactobacillus* strains, the pH values were 1, 2, 3 and 4. Hydrochloric acid (2 M) was used to adjust the pH of the PBS. For *Lact. acidophilus* I 26 and *Lact. fermentum* I 25, the tubes were incubated for 0, 0·5, 1, 2, 3 and 4 h and for the rest of the *Lactobacillus* strains, for 0, 0·5, 1, 2 and 3 h. Three tests, each with a duplicate, were made on each strain at each pH value and incubation period. After the incubation period, 0·1 ml from each tube was cultured on MRS agar plates and viable bacterial colonies counted.

RESULTS AND DISCUSSION

The results show that bile exerted a slight inhibitory effect on the growth of 10 *Lactobacillus* strains but two strains, *Lact. brevis* I 23 and *Lact. fermentum* I 24, were not affected. The delay in growth (d) caused by bile was 0·6 min for *Lact. brevis* I 218, 5·4 min for *Lact. acidophilus* I 26, 7·8 min for *Lact. brevis* I 211, 13·8 for *Lact. fermentum* C16, 14·4 min for *Lact. brevis* C 10, 15·0 min for *Lact. brevis* C 17, 16·2 min for *Lact. crispatus* I 12, 29·4 min for *Lact. acidophilus* I 16, 30·0 min for *Lact. fermentum* I 25 and 37·2 min for *Lact. brevis* C1. Detailed comparisons of growth rates of the *Lactobacillus* strains in the control and 3% bile cultures for 6 h also revealed that bile had only a slight inhibitory effect.

Resistance to bile is an important characteristic that enables *Lactobacillus* to survive and grow in the intestinal tract. Gilliland *et al.* (1984) reported that supplementation of the diet with the more bile-resistant strain of *Lact. acidophilus* increased the numbers of the strain in the upper small intestine. Chateau *et al.* (1994) arbitrarily classified bile resistance of *Lactobacillus* into four groups: resistant strains (delay of growth $d \leqslant 15$ min), tolerant strains (15 min $< d \leqslant 40$ min), weakly tolerant strains (40 min $< d <$ 60 min) and sensitive strains ($d \geqslant 60$ min). According to this classification, all 12 *Lactobacillus* strains tested in the present study are either resistant (seven strains) or tolerant (five strains). Therefore, it is likely that all 12 *Lactobacillus* strains are able to tolerate bile in the chicken intestine. As this is the first report on bile tolerance of *Lactobacillus* strains from chicken, there are no other data available for comparison.

The results on acid tolerance (as survival at various pH levels) showed that most of the *Lactobacillus* strains were tolerant to acid. The survival of *Lact. acidophilus* I 26 and *Lact. fermentum* I 25 (ileal strains which had strong ability to adhere to ileal epithelial cell; Jin *et al.* 1996) was very similar. Survival rates of *Lact. acidophilus* I 26, as a representative of the two strains, are shown in Table 1. Generally, survival was low at pH 0·5, 1·0 and 2·0, moderate at pH 3·0 and good at 4·0 and 5·0.

Survival of the six ileal strains (*Lact. fermentum* I 24, *Lact. crispatus* I 12, *Lact. brevis* I 211, *Lact. brevis* I 218, *Lact. brevis* I 23, *Lact. acidophilus* I 16) which showed moderate ability to adhere to intestinal cells (Jin *et al.* 1996) was similar except that *Lact. brevis* I 23 and *Lact. acidophilus* I 16 had a moderate survival rate (4·5–5·5 $\log_{10}$ cfu ml^{-1}) for 0·5 h at pH 2·0, while the other four strains had a very low survival rate ($<$1·0 $\log_{10}$ cfu ml^{-1}) at pH 2·0. However, all six strains had a moderate survival rate at pH 3·0 and a good survival rate at pH 4·0. Survival of these strains, as represented by *Lact. fermentum* I 24, is presented in Table 1.

Lactobacillus strains from the caecum (*Lact. brevis* C 1, *Lact. brevis* C 10, *Lact. fermentum* C 16, *Lact. brevis* C 17) showed better tolerance to acid than those from the ileum. Most caecal *Lactobacillus* strains could survive even at a low pH of 1·0 for up to 2 h of incubation, and all strains showed a moderate survival rate at pH 2·0, and good survival rate at pH 3·0 and 4·0. Table 1 shows the survival of *Lact. brevis* C 10 as a representative of the caecal strains.

In comparison to humans and domestic animals such as pigs and cattle, the alimentary tract of chicken is shorter. The time required for feed to pass through the entire alimentary canal is as short as 2·5 h (Duke 1977). Therefore, acid tolerance for bacterial strains in chickens is not as crucial as for

Table 1 Survival of *Lactobacillus acidophilus* I 26, *Lact. fermentum* I 24 and *Lact. brevis* C 10 at various pH values as determined by counts of viable bacteria

		Viable bacteria ($\log_{10}$ cfu ml^{-1})					
Strain	pH	0 h	0·5 h	1·0 h	2·0 h	3·0 h	4·0 h
Lact. acidophilus	0·5	7·1 ± 0·20	2·8 ± 0·10	< 1 ± 0	< 1 ± 0	< 1 ± 0	< 1 ± 0
I 26 (from ileum)	1	7·3 ± 0·15	2·8 ± 0·10	< 1 ± 0	< 1 ± 0	< 1 ± 0	< 1 ± 0
	2	7·0 ± 0·18	7·0 ± 0·21	< 1 ± 0	< 1 ± 0	< 1 ± 0	< 1 ± 0
	3	7·2 ± 0·21	7·1 ± 0·12	6·0 ± 0·25	4·3 ± 0·14	4·8 ± 0·23	4·0 ± 0·09
	4	7·5 ± 0·28	7·0 ± 0·08	7·0 ± 0·09	7·0 ± 0·21	6·7 ± 0·14	6·8 ± 0·07
	5	7·3 ± 0·25	7·0 ± 0·19	7·2 ± 0·12	7·1 ± 0·18	7·1 ± 0·18	7·0 ± 0·27
Lact. fermentum	1	7·3 ± 0·26	< 1 ± 0	< 1 ± 0	< 1 ± 0	< 1 ± 0	*
I 24 (from ileum)	2	7·3 ± 0·20	< 1 ± 0	< 1 ± 0	< 1 ± 0	< 1 ± 0	*
	3	7·5 ± 0·18	—	6·1 ± 0·21	3·2 ± 0·19	2·2 ± 0·24	*
	4	7·3 ± 0·26	—	7·4 ± 0·25	7·2 ± 0·21	6·9 ± 0·27	*
Lact. brevis C 10	1	7·3 ± 0·21	3·6 ± 0·12	2·9 ± 0·06	2·9 ± 0·06	—	*
(from caecum)	2	7·6 ± 0·25	7·5 ± 0·21	6·0 ± 0·19	6·0 ± 0·19	4·6 ± 0·01	*
	3	7·5 ± 0·20	—	7·5 ± 0·25	7·5 ± 0·25	6·0 ± 0·14	*
	4	7·5 ± 0·30	—	7·6 ± 0·21	7·6 ± 0·21	6·6 ± 0·24	*

Values are the means of three experiments, each with a duplicate; ±, standard error of mean; —, not detected.
* Not conducted.

those in other animals where the feed passage rate is much longer. However, the pH of the gastric juice in chickens can be as low as 0·5–2·0. It can be inferred that all caecal strains and some ileal strains such as *Lact. acidophilus* I 26, *Lact. fermentum* I 25, *Lact. brevis* I 23 and *Lact. acidophilus* I 16 could possibly pass through the crop and gizzard and survive in the small intestine, but some of the other ileal strains may not be able to survive the passage through the crop and gizzard and reach the intestine because of their weak tolerance to low pH.

From this study, it can be concluded (i) that all the 12 *Lactobacillus* strains were resistant or tolerant to 0·3% chicken bile salts, and (ii) most of the *Lactobacillus* strains were tolerant to acid but, generally, the caecal strains were more tolerant than the ileal strains.

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