TECHNICAL NOTES

Importance of Bile Tolerance of *Lactobacillus acidophilus* Used as a Dietary Adjunct[1]

S. E. GILLILAND,[2] T. E. STALEY,[3] and L. J. BUSH[2]
Oklahoma State University
Stillwater 74078

ABSTRACT

Cultures of lactobacilli identified as *Lactobacillus acidophilus* from the intestinal contents of young calves varied in their ability to grow in broth containing .3% oxgall compared with control broth. Frozen concentrated cultures were prepared from a strain exhibiting low tolerance to bile and from a strain exhibiting high tolerance to bile. Plate counts were comparable from the concentrated cultures before and after frozen storage on lactobacilli MRS agar with and without .15% oxgall. In a feeding trial involving newborn dairy calves supplementation of the diet with the more bile resistant strain of *Lactobacillus acidophilus* caused greater increases of numbers of facultative lactobabilli in the upper small intestines than did the strain exhibiting lower resistance to bile. It was not possible to determine whether the lactobacilli would prevent intestinal infections in the calves challenged with enteropathogenic *Escherichia coli.* This portion of the study failed as the challenge with *Escherichia coli* did not cause infections even in control animals.

INTRODUCTION

The use of *Lactobacillus acidophilus* as a dietary adjunct can provide several benefits to the digestive system (2, 12, 13, 14). The most frequently mentioned role for this organism is its role in helping to control undesirable microorganisms in the intestinal tract. Unfermented milk containing cells of *L. acidophilus* provides a source of these organisms that may be helpful in preventing intestinal infections (14). Its presence in milk is also beneficial to those individuals who cannot digest lactose adequately (9). The bacterial cells serve as a source of an enzyme system for hydrolyzing lactose in the intestinal tract. The organism may be useful in helping control blood serum cholesterol (7).

Lactobacillus acidophilus possesses several characteristics that enable it to survive and grow in the intestinal tract. Among these is the ability to grow in the presence of bile. This characteristic has been identified as important to maintain in preparation and storage of concentrated cultures for use as dietary adjuncts (2). Although bile resistance is important, information is lacking on the degree of bile resistance necessary to enable *L. acidophilus* to grow most effectively in the intestinal tract.

One objective of this study was to compare the ability of two strains of *L. acidophilus* exhibiting different degrees of bile resistance to increase the number of facultative lactobacilli in the intestinal tract. A second objective was to determine if consumption of unfermented milk containing cells of *L. acidophilus* would prevent intestinal infection caused by enteropathogenic *Escherichia coli.*

MATERIAL AND METHODS

Source and Maintenance of Cultures

Cultures of *L. acidophilus* in these experiments were isolated from intestinal contents of 2- to 5-wk-old calves. Fecal or intestinal contents were plated on lactobacillus selection (LBS) agar (BBL Microbiological Systems, Becton Dickinson Co.) and incubated 48 h at 37°C in a carbon dioxide enriched atmosphere. (The LBS agar was prepared from individual ingredients according to manufacturer's directions.) Isolated colonies were picked into lactobacilli MRS broth (Difco) and

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[2]Animal Science Department.
[3]Department of Physiological Sciences.

incubated at 37°C. Cultural characteristics used to identify isolates included: Gram-stain reaction, ability to grow at 15°C, and biochemical reactions by the BBL Minitek system as described by Gilliland and Speck (5). Identification was based on characteristics of *L. acidophilus* presented in the 8th edition of *Bergey's Manual of Determinative Bacteriology* (1). Cultures were maintained by propagation in sterile 10% nonfat milk solids (10% wt/vol in water) supplemented with .5% thiotone (BBL) with 1% inocula and 18-h incubation at 37°C. Cultures were stored in a refrigerator at 3 to 5°C between transfers.

Enteropathogenic *Escherichia coli* B44 (serotype 09:K30:NM) was obtained from L. L. Myers (Veterinary Research Laboratory, Montana State University, Bozeman). The culture was maintained by propagation on trypticase soy agar (BBL) slants with incubation for 18 h at 37°C. It was stored at 3 to 5°C between transfers.

Comparison of Bile Tolerance

Lactobacilli MRS broth was prepared with and without .3% oxgall (BBL) dispensed in 10-ml volumes and sterilized by autoclaving at 121°C for 15 min. For each culture to be tested, one tube of each media was inoculated with .1 ml of a freshly prepared lactobacilli MRS broth culture. The inoculated media were incubated at 37°C in a water bath. Growth was monitored by increases of A_{600nm} with a Spectronic 21 colorimeter. Growth curves were plotted and times required for turbidity to reach an optical density of .3 determined.

Preparation of Frozen Concentrated Cultures

Four liters of MRS broth was inoculated with 40 ml of a freshly prepared MRS culture of the desired strain of *L. acidophilus* and incubated 18 h at 37°C. Bacterial cells were harvested by our centrifuging the culture at 4000 × *g* for 20 min at 0°C. The cell pellets were resuspended in twice their weight of cold sterile 10% nonfat milk solids and dispensed in 2-g quantities into sterile cryogenic vials, then frozen and stored in liquid nitrogen. The population of *L. acidophilus* and its storage stability were ascertained by appropriate dilutions of the concentrated cultures plated on MRS and MRSO agar (MRS agar plus .15% oxgall) before and after frozen storage (10).

Preparation of Challenge Doses of *Escherichia coli*

Enteropathogenic *E. coli* B44 was surface inoculated onto Minca IsoVitalex agar as described by Guinee et al. (8) and incubated 18 h at 37°C. Bacterial cells were washed from the surface of the agar medium with sterile physiological saline solution. The cell suspension was adjusted to a population of 10^7 cells/ml. Each challenge dose was prepared just prior to use, packed in ice water, and transported to the Dairy Cattle Center. Ten milliliters of the challenge dose was administered to each calf.

Feeding Trials

Newborn Holstein and Ayrshire bull calves (16) were isolated from their dams immediately after birth and placed in individual stalls so that calves receiving the same treatment were not adjacent. Calves were assigned randomly within blocks of four to four treatment groups. Treatments included, unchallenged control, challenged control (with *E. coli*), *L. acidophilus* C28 (challenged with *E. coli*), and *L. acidophilus* 27SC (challenged with *E. coli*). Each calf was fed twice daily an amount of raw mixed herd milk equal to 11.25% of its metabolic size. The raw milk was obtained fresh daily from the bulk refrigeration tank at the Oklahoma State University Dairy Cattle Center and held in the refrigerator at 2 to 4°C. The two control groups received milk without any addition. Sufficient concentrated culture of the appropriate strain of *L. acidophilus* was added to the milk of the remaining groups to yield a total population of 10^{11} viable cells just prior to each feeding. The concentrated cultures were stored on dry ice at the Dairy Cattle Center for not more than 7 days. Immediately prior to feeding, sufficient frozen concentrated culture was thawed by submerging the vial in 1 liter of tap water at approximately 27°C. The necessary amount of the culture then was mixed into the appropriate amount of raw milk for each calf. The milk was fed via nursing bottles, which were washed and sterilized prior to each use. At the fifth or sixth feeding (morning feeding), each calf, except those in the unchallenged control group, was challenged with 10^8 cells of enteropatho-

genic *E. coli* B44. The cells of *E. coli* were added to the appropriate milk just prior to feeding. Twenty-four hours after challenge, each calf was transported to the veterinary clinic and sacrificed (3). Each calf in the unchallenged control group also was sacrificed 24 h after the fifth or sixth feeding. Approximately 10- to 15-cm segments of the midjejunum, terminal ileum, and midlarge intestine were removed surgically (3), placed in sterile containers, packed in ice, and transported to the microbiology laboratory for analyses. The numbers of facultative lactobacilli and coliforms were enumerated by procedures of (6).

Statistical Analyses

Data from each intestinal segment were analyzed separately. Each set of data for both types of microbial counts was analyzed as a randomized block design according to procedures in Steel and Torrie (15). Sources of variation tested were blocks and treatments. Least significant difference analyses were used to compare treatment means for which the analysis of variance indicated significant ($P<.05$) variation.

RESULTS

Identity characteristics of isolates are in Table 1. The seven isolates were identified as *L. acidophilus,* based on characteristics described for this species in the 8th edition of *Bergey's Manual of Determinative Bacteriology* (1). There were minor differences in fermentation patterns of the strains; however, the identity of each more closely matched characteristics of *L. acidophilus* than any other species of lactobacilli described in this reference. Strains C28, 27SC, and 36SB differed from characteristics of *L. acidophilus* in that they did not ferment trehalose. Strain 30SC differed in that it did not ferment amygdalin, salicin, or trehalose.

TABLE 1. Identity characteristics of lactobacilli isolated from intestinal contents of young calves.

	Strain							
Test	Bergey[1]	C28	30SC	27SC	25SB	36SB	FR 1	FR 2
Gram-positive rod	+	+	+	+	+	+	+	+
Catalase	−	−	−	−	−	−	−	−
Growth at 15°C	−	−	−	−	−	−	−	−
Ammonia from arginine	−	−	−	−	−	−	−	−
Hydrolysis of esculin	+	+	+	+	+	+	+	+
Fermentation of:								
Amygdalin	+	+	−	+	+	+	−	−
Arabinose	−	−	−	−	+	−	−	−
Cellobiose	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	−	+
Mannitol	−	−	−	−	−	−	−	−
Mannose	+	+	+	+	+	+	+	+
Melezitose	−	−	−	−	−	−	−	−
Melibiose	±	+	+	+	+	+	−	−
Raffinose	±	+	+	+	+	+	−	−
Rhamnose	−	−	−	−	−	−	−	−
Salicin	+	+	−	+	+	+	−	−
Sorbitol	−	−	−	−	−	−	−	−
Sucrose	+	+	+	+	+	+	+	+
Trehalose	+	−	−	−	−	−	+	+
Xylose	−	−	−	−	−	−	−	−

[1]Characteristics of *Lactobacillus acidophilus* as reported in the 8th edition of *Bergey's Manual of Determinative Bacteriology.*

TABLE 2. Comparison of bile tolerance of strains of *Lactobacillus acidophilus* isolated from intestinal contents of calves.

Strain	Hours to reach A_{600nm} = .3	
	MRS Broth	MRS Broth +.3% oxgall
C28	2.62[1]	>6
30SC	3.67	5.60
27SC	2.37	2.82
25SB	2.17	2.62
36SB	2.38	2.72
FR 1	3.02	>6
FR 2	3.50	>6

[1] Average time from three trials.

Strain 25SB differed in that it fermented arabinose but did not ferment trehalose. Strains FR 1 and FR 2 differed in that they did not ferment amygdalin or salicin. Also strain FR 1 did not ferment maltose. Although all strains were identified *L. acidophilus*, strains C28, 27SC, and 36SB most closely matched identity characteristics of *L. acidophilus*.

Comparison of the ability of the seven strains of lactobacilli to grow in the MRS broth with and without .3% oxgall revealed considerable variation among strains (Table 2). When compared with the control broth, .3% oxgall exerted inhibitory effect on all strains. Strains 27SC, 25SB, and 36SB were the most resistant strains. Strains C28, FR 1, and FR 2 had not reached an absorbance of .3 at 6 h. Because of identity characteristics and tolerance to bile, strain C28 was selected as one with low tolerance to bile and strain 27SC as one with high tolerance to bile for comparison in the feeding trial. A more complete comparison of the growth of these two strains in MRS broth with and without .3% oxgall is in Figure 1 in which the A_{600nm} is plotted against incubation time. For both cultures .3% oxgall appeared to limit maximum growth.

Viability of selected strains of *L. acidophilus* in concentrated cultures was not affected by freezing and storing the cultures in liquid nitrogen. For both strains C28 and 27SC the plate counts on MRS agar and MRSO agar was the same before and after 30 days of storage in liquid nitrogen (−196°C). Removal of the cultures from liquid nitrogen followed by storage on dry ice (−76°C) also had no effect on counts on the MRS and MRSO agar media. The populations of *L. acidophilus* in the frozen concentrated cultures were in the range of 2×10^{10}/g to 3×10^{10}/g.

Challenging the calves with enteropathogenic *E. coli* in the feeding trial was unsuccessful. None of the animals receiving the challenge dose developed any symptoms normally associated with infections caused by this organism. There was no evidence of scouring. Furthermore, the challenge had no significant influence on the numbers of coliform organisms in any of the intestinal segments examined (Table 3). Numbers of coliforms are presented as the $\log_{10}$/gram dry weight. Data are presented only for control and control challenge groups. Supplementation of the raw milk with either *L. acidophilus* C28 or 27SC had no significant

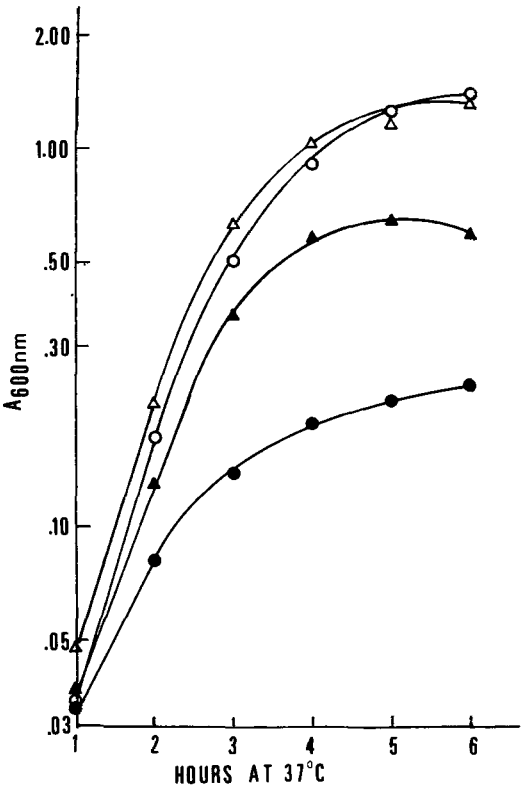

Figure 1. Growth of *Lactobacillus acidophilus* C28 and 27SC in lactobacilli MRS broth with and without .3% oxgall (△ strain 27SC control; ▲ strain 27SC with .3% oxgall; ○ strain C28 control; ● strain C28 plus .3% oxgall).

TABLE 3. Influence of challenging calves with enteropathogenic *Escherichia coli* B44 on numbers of coliforms in jujunum, ileum, and large intestines.

Treatment	Log_{10} coliform/gram dry weight					
	Jejunum		Ileum		Large intestine	
	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
Control	5.25	1.26[1]	6.59	.79	7.45	.98
Challenged	5.19	.58	6.61	1.07	8.05	1.37

[1]Averages from seven calves per group; no significant differences between groups for any of the intestinal locations ($P>.05$).

influence on numbers of coliforms in any of the intestinal segments (data not shown).

Comparisons of the influence of supplementing milk with the two strains of *L. acidophilus* upon numbers of facultative lactobacilli in the intestinal tract are in Table 4. Data are mean log_{10}/gram dry weight of intestinal material for each segment sampled. So that the only factor compared was presence or absence of the two strains of *L. acidophilus*, only data for the three treatment groups that were challenged with the *E. coli* are included in the table. In the jejunum, numbers of facultative lactobacilli appeared to be greater in both groups of calves that received *L. acidophilus*; however, the difference was significant for only the group that received *L. acidophilus* 27SC ($P<.05$). In the ileum, numbers were significantly higher ($P<.05$) for groups of calves receiving both strains of *L. acidophilus* than for the control group. The difference between groups receiving the two strains of *L. acidophilus* was not significant ($P>.05$). In the large intestine although numbers of lactobacilli appeared to be greater in both groups that received *L. acidophilus*, neither was significantly greater than in control group. In all groups numbers of lactobacilli were higher in the large intestine than in either jejunum or ileum.

DISCUSSION

Escherichia coli B44 was effective as a challenge organism for causing intestinal infection resulting in scours in calves (11). Thus, it was disappointing that in our study it was ineffective. To establish an infection in calves that have not been fed colostrum is easier. For this reason, none of the calves

TABLE 4. Influence of feeding cells of low and high bile tolerant *Lactobacillus acidophilus* on numbers of facultative lactobacilli in intestines of dairy calves.

Treatment[2]	Bile resistance[3]	Log_{10} count/gram dry weight[1]					
		Jejunum		Ileum		Large intestine	
		$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
Control	...	5.77	1.14[a]	5.57	.93[a]	6.91	1.47
L. acidophilus C28	Low	6.57	.60	7.00	1.37[b]	7.87	.96
L. acidophilus 27SC	High	7.07	.76[b]	6.79	.52[b]	8.24	.65

[a,b]Means in same column followed by different superscripts differ ($P<.05$).

[1]Mean from seven calves.

[2]Calves in all treatments were challenged with *E. coli* B44 24 h prior to being sacrificed.

[3]Bile resistance based on comparison of culture growth in broth with and without .3% oxgall.

received colostrum. Inability to produce the infection could have been from inadequate numbers of *E. coli* in the challenge dose. Another factor could have been differences in development of the natural flora in calves. To confirm whether consumption of milk supplemented with *L. acidophilus* can be effective in preventing intestinal infections, it will be necessary to conduct experiments in which a successful challenge is included.

As might be expected, bile tolerance among strains of *L. acidophilus* varied. Agar media containing .15% oxgall is useful to enumerate lactobacilli resistant to bile (4) and to study maintenance of resistance to bile of the cultures exposed to different treatments and during frozen storage (10). The use of such agar media, however, does not permit comparison of rapidity of growth of cultures in the presence of bile, since comparisons are made on the basis of numbers of colonies formed rather than growth rates. Our method not only permits comparison of relative growth rates of cultures with and without bile, but by increasing oxgall to where all strains are inhibited at least partially provides a more sensitive test for comparing tolerance to bile. Thus, it may be a more useful test for screening cultures for resistance to bile than would be the ability to grow on the agar medium containing .15% oxgall. Resistance to bile of *L. acidophilus* is an important characteristic that enables it to survive and grow in the intestinal tract (2). Degree of resistance to bile of the cultures is important. Of the two strains tested in the feeding trial, the one that was more tolerant to bile enabled greater numbers of facultative lactobacilli in the upper part of the small intestine (jejunum) than did the less resistant strain. The concentration of bile salts might be expected to be higher in this segment of the intestine than in the ileum because it is closer to where bile enters the intestinal tract. No difference in numbers of lactobacilli was significant in the ileum in the groups receiving the two strains of *L. acidophilus* although both strains caused significantly higher numbers than control groups. Perhaps the bile concentration was not as great in the ileum as in the jejunum. The ability of *L. acidophilus* to cause significant increases of numbers of lactobacilli in the upper portion of the small intestine may be critical for controlling growth of intestinal pathogens entering the digestive system.

In all treatment groups there was a trend toward more lactobacilli in the large intestine than in either segment of the small intestine. Although differences among treatment groups were not significant, there was also a trend toward more lactobacilli in the large intestine for those calves receiving milk containing *L. acidophilus* than for the control group. Furthermore, numbers appeared to be largest in the group that received the more resistant strain of *L. acidophilus.* Thus, numbers of facultative lactobacilli that appear in the large intestine and perhaps in the fecal material might be used as an indication of relative numbers of lactobacilli in the upper intestinal tract.

In summary, degree of tolerance to bile exhibited by *L. acidophilus* is important in governing its effectiveness in growing in the intestinal tract, particularly in the upper small intestine. Additionally, it is possible to supplement raw milk with the concentrated culture of *L. acidophilus* to increase numbers of facultative lactobacilli in young dairy calves. In previous studies, pasteurized milk has been used as a carrier for cells of *L. acidophilus.*

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