INFLUENCE OF BILE ON CELLULAR INTEGRITY AND β-GALACTOSIDASE ACTIVITY OF *LACTOBACILLUS ACIDOPHILUS*

BY

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CHAPTER I

INTRODUCTION

Lactose intolerance or lactose maldigestion, caused by reduced lactase activity in the intestine, is common in most populations. Diarrhea, flatulence and abdominal cramping are major symptoms caused by lactose intolerance. Because of these symptoms, many people avoid milk consumption.

One method introduced to reduce the lactose intolerance is the addition of lactase, an enzyme hydrolyzing lactose into glucose and galactose, to milk. With this enzyme, the lactose in milk is hydrolyzed prior to consumption, resulting in reduction or prevention of the symptoms associated with lactose intolerance. However, this product has a sweeter taste and is more expensive than regular milk, so it may not be widely acceptable to many people.

Another approach to improving lactose utilization by persons suffering from lactose maldigestion relies on viable lactic acid bacteria in cultured or culture-containing milk products. One of those beneficial bacteria is Lactobacillus acidophilus, a normal inhabitant of the small intestine. L. acidophilus has been used increasingly as a dietary adjunct since Metchnikoff first mentioned its desirability in the intestinal tract. Because it contains β-galactosidase, L.

acidophilus is a good dietary adjunct for lactose maldigestors. It has been shown to improve lactose utilization in such persons. Several characteristics of L. acidophilus are beneficial for use as a dietary adjunct. It is capable of surviving and growing in the intestine. Furthermore, it can resist gastric acid in stomach and bile salts in the intestine.

In order for a microorganism to be a good dietary adjunct for improving lactose utilization, the cells should survive in the intestinal tract and contain high β-galactosidase activity. L. acidophilus is bile-tolerant which is important in surviving in the intestinal tract. The β-galactosidase activity of L. acidophilus is increased in the presence of oxgall(dehydrated bile) in vitro. However, the mechanism whereby bile increases β-galactosidase activity in L. acidophilus has not been reported.

The purpose of this study was to investigate the degree of bile tolerance and the influence of the presence of bile on β-galactosidase activity and cellular integrity of different strains of L. acidophilus. With the results from this study, it may be possible to determine the most appropriate strain to be used as a dietary adjunct in milk product to best reduce lactose maldigestion.

CHAPTER II

REVIEW OF LITERATURE

Gilliland (1989) reported that consumption of dairy products containing L. acidophilus has the potential of controlling intestinal infections, providing anticarcinogenic activity, improving lactose utilization and finally, aiding in controlling serum cholesterol levels.

Gilliland and Speck (1977a) reported that intestinal lactobacilli can deconjugate bile acids. The liberation of free bile acids in the intestinal tract could influence the balance of bacterial species present there. Floch et al. (1972) showed that, compared with the conjugated forms, deconjugated bile acids had greater inhibitory effect on bacteria and controlled the intestinal bacterial populations in vivo.

With these beneficial characteristics, L. acidophilus has been used increasingly as a useful dietary adjunct for human consumption. This increased use of L. acidophilus required the need for preparation of cultures that retain viability, are resistant to bile and are capable of survival in the intestinal tract (Speck,1980). At first, L. acidophilus was available only in fermented milk product.

However, this product had undesirable flavor, so it was not widely accepted.

Among microorganisms, cells of L. acidophilus have been shown to be appropriate dietary adjuncts for human consumption (Gilliland,1979). L. acidophilus shows antagonistic action toward undesirable microorganisms and they do not grow under 15^{0}C so that they would not grow during storage of the refrigerated nonfermented acidophilus milk. There is no hydrolysis of lactose during storage of the milk (Kim and Gilliland, 1983). In addition, they also exhibit bile tolerance which is an important factor to permit their survival and growth in the small intestine.

There are several terms referring to the inability of person to digest lactose, such as "lactose intolerance", "lactose malabsorption", and "lactose maldigestion". Among them, probably "lactose maldigestion" is the most accurate term to describe this symptom, since it refers to the inability of the person to digest the lactose adequately.

Lactose maldigestion, which is experienced by many people around the world, is caused by lack of sufficient enzyme activity in the small intestine to hydrolyze the lactose. According to Sandine and Daly (1979), diarrhea, flatulence and abdominal cramping are the major symptoms resulting from lactose maldigestion. Due to these symptoms, people having lactose maldigestion tend to avoid the consumption of milk thus deleting a major source of protein and calcium from the diet.

Several ways to avoid the problems associated with lactose maldigestion have been introduced and used. One of them is to utilize microorganisms containing β-galactosidase as dietary adjuncts for milk. Nonfermented acidophilus milk is the product prepared by the addition of a concentrated suspension of cells of lactobacilli to pasteurized milk which is then kept refrigerated. There is no hydrolysis of lactose during storage, since cells do not grow under refrigeration. This product tastes the same as the pasteurized milk, so it is more likely to be accepted by some lactose maldigestors than would fermented products (Gilliland,1989).

Kim (1981) reported that consuming nonfermented pasteurized whole milk containing cells of L. acidophilus NCFM significantly reduced the lactose maldigestion, which was monitored by measuring a reduction in the amount of hydrogen excreted in the breath of test subjects. In their recent experiment, Lin et al. (1991) also reported that nonfermented yogurt milk containing either cells of Streptococcus thermophilus or L. bulgaricus and nonfermented acidophilus milk containing cells of L. acidophilus LA-1 decreased lactose maldigestion significantly.

Conversely, there are other reports showing that L. acidophilus showed no effects on lactose maldigestion (Newcomer et al., 1983; Payne et al., 1981; Savaiano et al., 1984). However, in those studies, there was little information regarding the culture used or the storage method

of the culture before testing. Therefore, it may be possible that there was inadequate β-galactosidase activity. Among them, Savaiano et al. (1984) reported that there was little, if any, β-galactosidase activity in the nonfermented acidophilus milk used in their study.

The cells of L. acidophilus can apparently enhance the hydrolysis of lactose in the intestine following consumption of nonfermented acidophilus milk (Kim & Gilliland, 1983; Lin et al., 1991). β-galactosidase is the enzyme responsible for the hydrolysis of lactose. This enzyme, being intracellular, is able to survive the passage through the stomach to reach the intestine. It has been purified and characterized from many microbial species (Demacias et al.,1983a, 1983b; Itoh et al.,1980; Miyazaki,1988; Nielsen,1987; Rymaszewski et al.,1985; Ramana and Dutta,1981). β-galactosidase from a number of different bacteria has been shown to possess common properties such as optimum temperature of 37^{0}C and optimum pH at 7.0.

Nielsen (1987) observed that β-galactosidase was present in 3 strains of L. acidophilus under study, while phospho-β-galactosidase was not. The activity of β-galactosidase purified from the cells also showed the highest activity at pH 6.6 and at 37^{0}C. In addition, the activity of the enzyme stored at 5^{0}C remained constant for the first four days, and decreased after. However, more than 50% of the original activity remained after 12 days of storage.

Nonfermented acidophilus milk has the appearance and taste of plain milk. It is prepared by adding a large quantity of cells of L. acidophilus as a concentrated culture to pasteurized cold milk. The microorganism should survive freezing and frozen storage for long period prior to use, since the nonfermented acidophilus milk is made by the addition of frozen concentrated cultures of bacteria to refrigerated pasteurized milk. Gilliland and Lara (1988) examined 3 strains of L. acidophilus for preparing nonfermented acidophilus that would benefit lactose maldigestors. They found that during storage at -196^{0}C for 28 days the viability of 3 strains did not show a decrease. However, the activity of β-galactosidase during subsequent storage at 5^{0}C, showed a significant decrease in only one of the three strains of L. acidophilus as storage time increased.

After being consumed, cells of lactobacilli should survive in the intestine, and exert high β-galactosidase activity to utilize more lactose. Kim (1981) reported that glucose content released from the hydrolysis of lactose increased as oxgall content was increased up to 1.25% in vitro, which means enzyme activity was enhanced in the presence of bile. He also reported that the increased β-galactosidase activity was not due to the increased number of cells but suggested it was due to the change in the permeability of cell membranes, allowing more lactose to enter the cells for hydrolysis.

The purpose of this study was to examine the influence of bile on the cells of lactobacilli and their ability to hydrolyze lactose.

CHAPTER III

MATERIALS AND METHODS

Media and Growth Conditions

Cultures were routinely maintained by weekly subculture in PMN (Peptonized Milk Nutrient) broth using 1% inocula and 18 hr incubation at 37^0C. Between transfers, they were stored in the refrigerator at 5^0C. Five strains of Lactobacillus acidophilus (107, 223, NCFM, 606 and 4356), all of human origin from the Dairy Microbiology Laboratory Culture Collection, were used in this study. The PMN broth contained all the following ingredients: 5% Peptonized Milk Nutrient (Sheffield Products); 2% Primatone (Sheffield Products); 0.1% Tween 80 (Sigma Chemical Co.); 0.1% yeast extract (Difco Laboratories);2% lactose (Difco Laboratories). After all ingredients were dissolved in distilled water, the broth was autoclaved for 15 minutes at 121^0C. The cultures were subcultured at least 3 times just prior to experimental use.

Bile Resistance of L. acidophilus

PMN broth was prepared with and without 0.3% oxgall (Difco Laboratories), dispensed in 10 ml volumes and sterilized by autoclaving at 121^0C for 15 min. For each

culture, one tube of each broth was inoculated with 0.1 ml of a freshly prepared PMN broth culture. The A_{620} was read with a Spectronic 21 spectrophotometer (Bausch & Lomb) against an uninoculated broth blank. The inoculated media were then incubated in a water bath at 37^0C. Growth was monitored by measuring the A_{620} every 30 minutes. The increase in A_{620} was plotted against time and the time required for the absorption to increase by 0.3 was determined.

Preparation of Cells for Measuring β-galactosidase Activity

The cells were harvested from 10 ml PMN broth culture by centrifugation (12,062 x g, 10 min., and 1-2^0C) in a Sorvall RC-5 Centrifuge (Dupont Co.). The cells were washed with 10 ml of cold 0.05 M sodium phosphate buffer (pH 7.0) and the washed cell pellets were resuspended in 10 ml of the same buffer.

Effect of Bile on β-Galactosidase Activity

In order to investigate the effect of bile on β-galactosidase activity, washed cells of the lactobacilli were assayed with (0.3% oxgall) and without oxgall. For the assay of enzyme activity, 0.012 M o-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma Chemical Co.) in 0.05 M sodium phosphate buffer (pH 7.0) was used as a substrate. Two test tubes were prepared for each culture, one with 3 ml

sodium phosphate buffer(0.05 M, pH 7.0) and one with 3ml of the same buffer plus 0.3% oxgall. Then, 2 ml of the cold ONPG reagent was added. One ml aliquots of each cell suspension were added to the appropriate tubes and incubated in a water bath at 37^0C for 10 min. After incubation, 4 ml of cold 0.625 M Na_2CO_3 were added to the mixtures to stop the reaction. Following mixing the mixtures were centrifuged for 10 minutes at 12,062 x g and $1-2^0$C to remove the cells. The clear supernatant was recovered and the A_{420} was read against a reagent blank. The calculation of micrograms of 0-nitrophenol (ONP) released was based on the relationship of the A_{420} to a standard curve. Activity was expressed as micrograms of ONP released per 10 min. of incubation.

Influence of Bile on Cellular Integrity

Two sets of test tubes, one with 3 ml phosphate buffer and one with 3 ml phosphate buffer plus 0.6% oxgall were prepared for each culture. Three ml of washed cell suspension was added to each tube and A_{620} was read. Then, the mixtures were placed in a water bath of 37^0C for incubation. The change in A_{620} was monitored every 10 minutes for 40 min. Any decrease of A_{620} was attributed to lysis of cells of the lactobacilli.

Influence of Bile on Cellular Retention of β-Galactosidase

To check the influence of bile on cellular retention of the enzyme, harvested and washed cells were incubated in the presence of bile prior to measuring the enzyme activity. Two sets of tubes, one containing 5 ml of 0.05 M phosphate buffer (pH7.0) plus 0.6% oxgall and one containing only 5 ml of 0.05 M phosphate buffer (pH 7.0), were prepared for each culture. Five ml of each cell suspension was added to one tube of each set. The final concentration of oxgall was 0.3% in the bile samples. The mixtures were incubated for 10 minutes at 37^0C for a pretreatment and then centrifuged for 10 minutes at 12,062 x g and $1\text{-}2^0$C. Clear supernatant was recovered and cell pellets were resuspended in 10 ml of cold sodium phosphate buffer. The supernatant recovered from each sample was checked for the presence of UV absorbing materials by reading A_{260} with DU-64 spectrophotometer (Beckman Instruments, Inc.). Both the supernatant and cell suspension for each culture were assayed for β-galactosidase activity as described before.

Enzyme Extraction and Measurement of Specific Activity

The lysis of cells with lysozyme was done using a modification of methods described by Metcalf and Deibel (1969). Cells were harvested from 10 ml of a freshly prepared PMN broth culture by centrifugation at 4,000 x g

for 10 minutes at 1^0C and washed three times with 10 ml portions of cold distilled water. The washed cells were resuspended in 16 ml of distilled water and transferred to test tubes. Two ml of 0.2% (w/v) lysozyme (Sigma Chemical Co.) solution were added to each cell suspension and the mixtures were incubated in water bath at 37^0C for 1 hour. After 1 hour, 2 ml of 4 M sodium chloride were added to each and then the tubes were inverted slowly 10 to 15 times for proper mixing. The lysed cells were centrifuged at 15,000 x g for 20 min. at 1^0C to remove cellular debris and the supernatant was collected.

The following solutions were prepared for enzyme assay: solution A (0.1 M sodium phosphate buffer, pH 7.3), solution B (3.36 M 2-mercaptoethanol), solution C (0.03 M magnesium chloride), and solution D (cell free supernatant containing the enzyme). According to DeMacias et al. (1983a,1983b), and Smart and Richardson (1987), mercaptoethanol acts as an activator for β-galactosidase and magnesium chloride stimulates and protects enzyme activity. The assay for the specific activity of β-galactosidase was similar to that reported by Rolfing and Crawford (1966). The substrate (0.068M ONPG) was prepared by dissolving 20.5 mg ONPG in 1.0 ml of solution A and holding in a 40^0C water bath until all ONPG was dissolved. For the assay of specific activity, first, 2.6 ml of solution A was dispensed into a 3 ml cuvette having a 1 cm light path. Then 0.1 ml aliquots of solution B, C,and D were added. The mixture was incubated

in water bath of 37^0C for 3 minutes to activate the enzyme. After that, 0.1 ml of the ONPG solution was added. The cuvette was then covered with parafilm and inverted quickly to mix the reagents. Then the change in A_{410} was monitored for 1 min with Spectronic 21 spectrophotometer (Bausch & Lomb) and the difference in A_{410} per minute was determined. The micromoles of ONP liberated were determined by multiplying the A_{410} by 3.5 which is the millimolar extinction coefficient of o- nitrophenol. One unit of enzyme activity was defined as micromoles of ONP liberated per mg of protein per minute at 37^0C.

The protein concentration in each sample was determined by the protein-dye binding method described by Bradford (1976). Bovine serum albumin (Bio-Rad Lab.) was used as protein standard.

Statistical Analyses

An analysis of variance was performed on each set of data to see if any significant differences existed. The differences and confidence levels were determined by calculating the least significant difference with SAS (1985).

CHAPTER IV

RESULTS

Bile Tolerance of L. acidophilus

All strains of L. acidophilus grew slower in PMN broth containing 0.3% oxgall than in PMN broth without oxgall (Table 1). However, the growth of only two strains , 223 and 4356, was significantly lower in the presence of oxgall ($P<.05$). The growth of the remaining 3 strains exhibited no significant differences from each other in the presence or absence of oxgall ($P>.05$). The two bile-sensitive strains (223 and 4356) grew significantly slower in the presence of bile than did the 3 others ($P<.05$). Strain 223 grew significantly slower in the control broth than did strains 107, NCFM, and 606 ($P<.05$)

Influence of Bile on β-Galactosidase Activity

β-galactosidase activity was significantly higher ($P<.05$) in the presence of oxgall for all 5 strains (Table 2). Among 5 strains, L. acidophilus 4356 showed the highest ($P<.05$) activity both in the absence and the presence of oxgall. Bile-tolerant strain 107 showed the highest increase of enzyme activity in the presence of oxgall. There were no ($P>.05$) differences in enzyme

TABLE 1.

BILE TOLERANCE OF DIFFERENT STRAINS OF LACTOBACILLUS ACIDOPHILUS

	Time[a]	
Strains	control	0.3% oxgall
107	4.2^{c}	4.8^{c}
223	5.0^{b}	7.2^{d}
NCFM	4.0^{c}	4.5^{c}
606	4.0^{c}	4.3^{c}
4356	4.8^{bc}	7.8^{d}

[a] hours for A_{620nm} to increase by 0.3 in PMN broth with and without 0.3% oxgall; each value is an average of 3 trials

b,c,d those with different superscript letters are significantly different($P<0.05$)

TABLE 2.

EFFECT OF BILE ON β-GALACTOSIDASE ACTIVITY* OF DIFFERENT STRAINS OF L. ACIDOPHILUS

Strains	No oxgall	0.3% oxgall
	ug ONP	ug ONP
107	20.79[a]	43.33[d]
223	29.70[b]	38.06[d]
NCFM	22.63[a]	38.54[d]
606	25.20[ab]	36.73[d]
4356	53.12[c]	59.65[e]

* enzyme activity(ug of ONP liberated); each value is an average of 3 trials

a,b,c,d,e those with different superscripts are significantly diffderent (P<.05)

activities in the presence of oxgall for the 3 remaining strains. Although the enzyme activities for all strains were increased significantly in the presence of oxgall, the increases did not appear to be related to the degree of bile-resistance.

Influence of Bile on Cellular Integrity

The effect of bile on cellular integrity was measured by monitoring the A_{620} of cell suspensions of each strain during incubation with and without added oxgall. The results are shown in Tables 3, 4, 5, 6, and 7. All strains exhibited some decrease in turbidity initially (time 0) when the cells were added to the buffer containing oxgall. All 5 strains exhibited decreases in A_{620} as incubation time increased both in the presence and absence of oxgall. However, there were no significant differences among the decreases in turbidity between two treatments for any of the 5 strains ($P>.05$). Therefore, it may be concluded that the presence of oxgall did not greatly enhance the lysis of cells of L. acidophilus.

Enzyme Extraction and Comparison of Specific Activity

In order to measure the specific activity, cells were lysed enzymatically and β-galactosidase activities were measured in the cell free extracts (Table 8). Strains 223 and 4356 had higher ($P<.05$) specific activities than the others.

TABLE 3.

EFFECT OF BILE ON CELLULAR INTEGRITY OF
LACTOBACILLUS ACIDOPHILUS 107

	A_{620nm}*	
Incubation time (min.)	No oxgall	0.3% oxgall
0	0.518	0.469
10	0.450	0.429
20	0.448	0.415
30	0.436	0.411
40	0.426	0.398

*Each value is an average of 3 trials

TABLE 4.

EFFECT OF BILE ON CELLULAR INTEGRITY OF
LACTOBACILLUS ACIDOPHILUS 223

	A_{620nm}*	
Incubation time (min.)	No oxgall	0.3% oxgall
0	0.552	0.473
10	0.469	0.432
20	0.462	0.424
30	0.456	0.415
40	0.450	0.409

*Each value is an average of 3 trials

TABLE 5.

EFFECT OF BILE ON CELLULAR INTEGRITY OF LACTOBACILLUS ACIDOPHILUS NCFM

	A_{620nm}*	
Incubation time (min.)	No oxgall	0.3% oxgall
0	0.495	0.443
10	0.433	0.413
20	0.433	0.399
30	0.421	0.384
40	0.414	0.378

*Each value is an average of 3 trials

TABLE 6.

EFFECT OF BILE ON CELLULAR INTEGRITY OF LACTOBACILLUS ACIDOPHILUS 606

	A_{620nm}*	
Incubation time (min.)	No oxgall	0.3% oxgall
0	0.519	0.469
10	0.473	0.426
20	0.463	0.409
30	0.456	0.398
40	0.448	0.387

*Each value is an average of 3 trials

TABLE 7.

EFFECT OF BILE ON CELLULAR INTEGRITY OF
LACTOBACILLUS ACIDOPHILUS 4356

	A_{620nm}*	
Incubation time (min.)	No oxgall	0.3% oxgall
0	0.516	0.467
10	0.467	0.436
20	0.452	0.424
30	0.444	0.422
40	0.444	0.417

*Each value is an average from 3 trials

TABLE 8.

COMPARISON OF SPECIFIC ACTIVITIES OF DIFFERENT STRAINS OF *LACTOBACILLUS ACIDOPHILUS*[a]

Strains	ug protein/ml	Units[b]	specific activity[c]
107	358.3	0.286	0.75[d]
223	294.7	0.714	2.43[e]
NCFM	323.0	0.229	0.71[d]
606	368.3	0.343	0.95[d]
4356	305.3	0.629	2.07[e]

a. average of three trials.
b. micromoles of ONP released per ml of protein per min.
c. specific activity = Units/mg protein.
d,e those with different superscripts are significantly different($P<.05$).

Influence of Bile on Cellular Retention of β-Galactosidase

Treatment of cells of the lactobacilli with oxgall had little effect on cellular retention of β-galactosidase (Table 9). For most of the cultures little, if any, enzyme activity was observed in the supernatant following removal of the bacterial cells whether or not the cells had been pretreated with oxgall. More enzyme activity was present in the supernatant fluids from strain 4356 than from any of the other four strains. Almost all enzyme activity for all strains remained with the cells.

Pretreatment with oxgall appeared to enhance the permeability of the cells for the substrate in that higher levels of activity were measured in the cells pretreated with oxgall compared to those not receiving the pretreatment.

The two bile-sensitive strains (223 and 4356) also showed more enzyme activities than other strains in control buffer and significant increases of activities when they were pretreated with oxgall($P<.05$).

Influence of Bile on Cellular Permeability

Leakage of intracellular material which absorbed light at 260nm occurred when cells of each strain were suspended in buffer containing 0.3% oxgall. The leakage increased with increased exposure time. Greater increases for all cultures occurred from cells suspended in buffer containing

TABLE 9.

EFFECT OF PRETREATMENT WITH BILE ON CELLULAR RETENTION OF β-GALACTOSIDASE OF LACTOBACILLUS ACIDOPHILUS[a]

Fraction	Strains	Control	Oxgall-Pretreatment
Supernatant	107	0.07	0.00
	223	0.12	0.99
	NCFM	0.09	0.04
	606	0.17	0.00
	4356	1.08	2.50
Cells	107	5.34[b]	7.21[b]
	223	27.18[c]	79.19[e]
	NCFM	4.54[b]	4.68[b]
	606	4.71[b]	13.41[b]
	4356	27.98c	47.90[d]

[a] enzyme activity is expressed as microgram of ONP liberated; each value is an average of 3 trials.

[b,c,d,e] those with different superscript letters are significant($P<.05$)

0.3% oxgall than in control buffer (Figures 1-5). Based on turbidity measurements (A_{620}) prior to removal of the cells, the density of cells was similar for all five cultures. The increased leakage of UV absorbing materials in the presence of oxgall indicates increased permeability of the cells. When the supernatants and cells were assayed for β-galactosidase activity after 60 min., the enzyme activity was detected only in cells for all strains (data not shown).

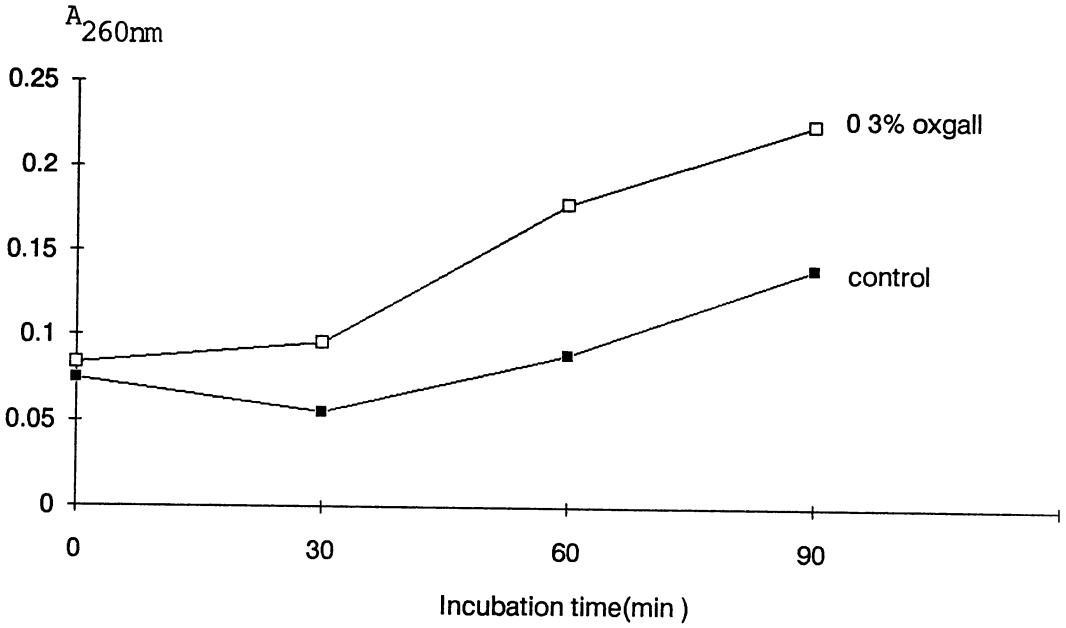

Fig. 1. Influence of Bile on leakage of intracellular materials from cells of Lactobacillus acidophilus 107

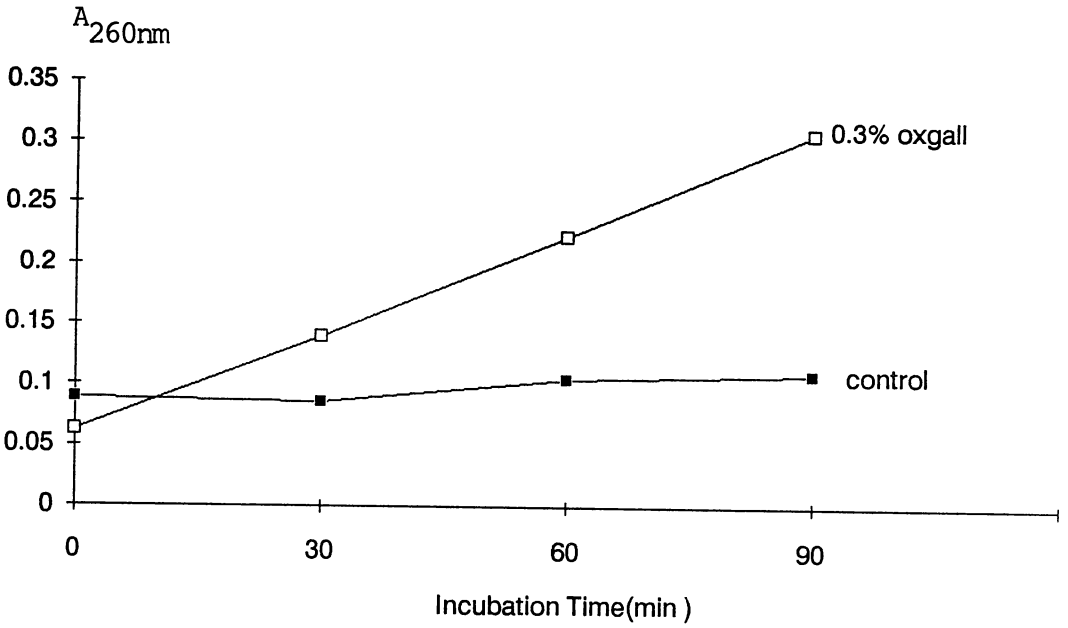

Fig. 2. Influence of Bile on leakage of intracellular materials from cells of Lactobacillus acidophilus 223

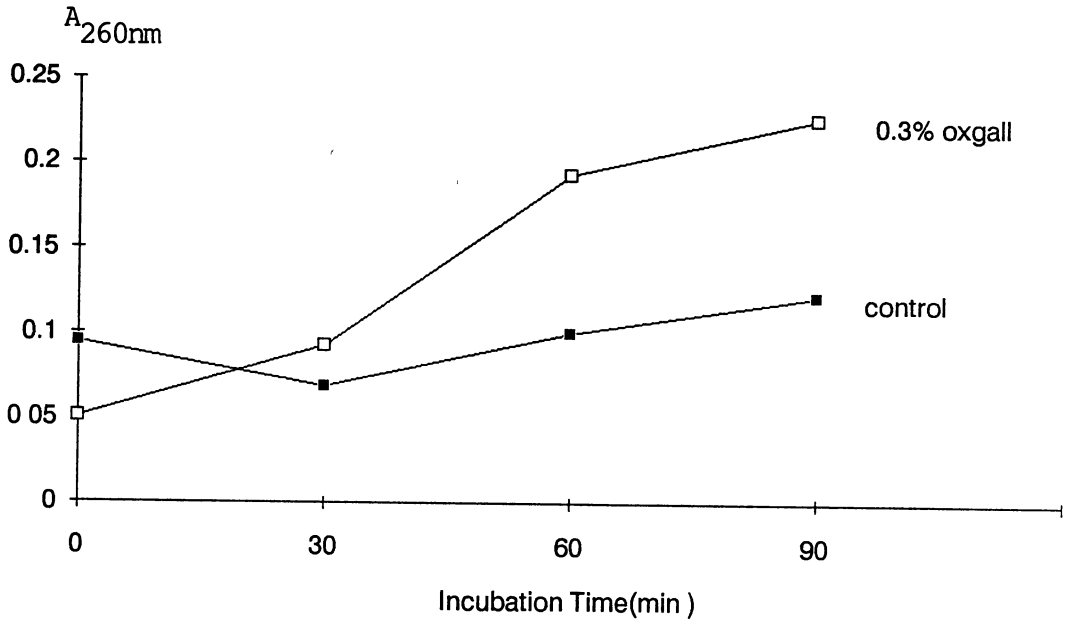

Fig. 3. Influence of Bile on leakage of intracellular materials from cells of Lactobacillus acidophilus NCFM

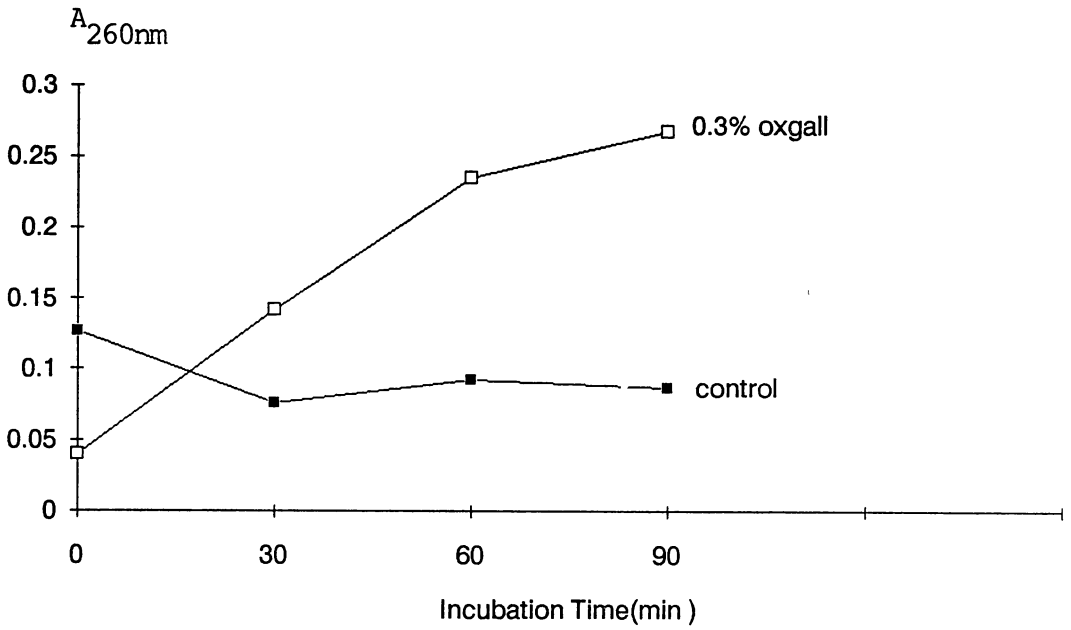

Fig. 4. Influence of Bile on leakage of intracellular materials from cells of Lactobacillus acidophilus 606

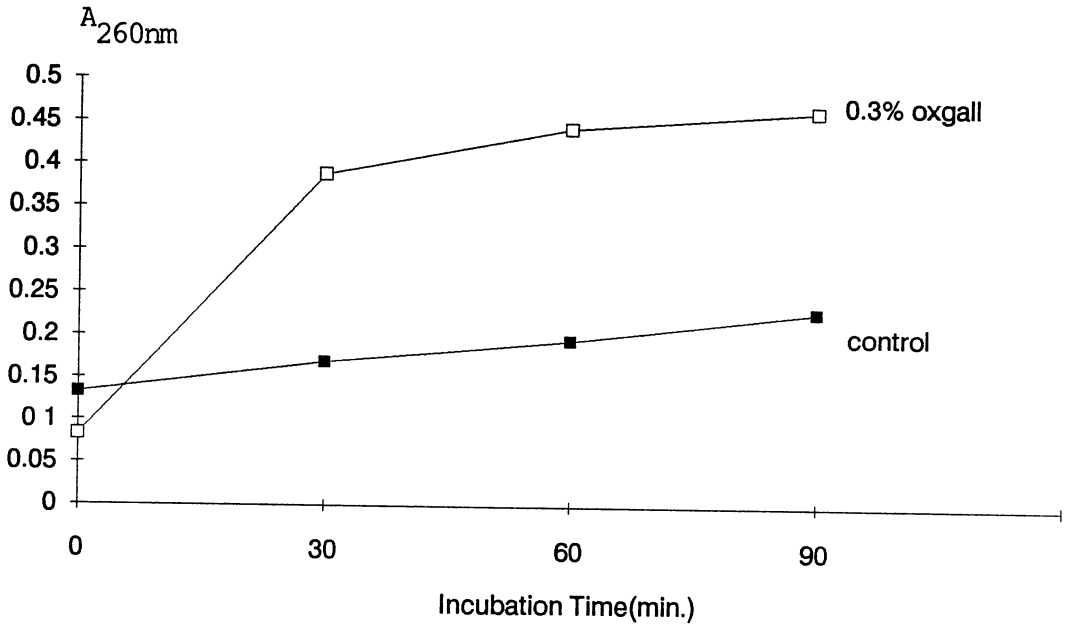

Fig. 5. Influence of Bile on leakage of intracellular materials from cells of Lactobacillus acidophilus 4356

CHAPTER V

DISCUSSION

Lactose maldigestion describes a condition in humans in which lactose cannot be adequately digested. This results from an insufficient amount of lactase in the small intestines to hydrolyze the lactose (Sandine & Daly, 1979). Because of the symptoms associated with this malady, lactose maldigestors usually avoid the consumption of milk.

Nonfermented milk containing added cells of Lactobacillus acidophilus has been shown to improve lactose utilization in persons classified as lactose maldigestors (Kim and Gilliland, 1983). L. acidophilus contains β-galactosidase which hydrolyzes lactose into glucose and galactose (Fisher et al.,1985) and by doing so, it reduces lactose maldigestion.

According to Speck (1980), consumption of live L. acidophilus may inhibit the growth of undesirable bacteria in gastro-intestinal tract in addition to helping those lactose maldigestors. Because of these beneficial roles, L. acidophilus has been of interest and is used in several dairy-based fermented products. However, these fermented milk products have displeasing flavors to some people. Thus they may not be readily accepted by some people who are

lactose maldigestors. Therefore, nonfermented dairy products with live cells of L. acidophilus may present a useful alternative.

Gilliland et al. (1978) reported that nonfermented acidophilus milk products could provide a source of L. acidophilus that would survive in the intestine.

Gilliland (1979) indicated that to survive and grow in the intestinal tract, microorganisms should be bile resistant. Since L. acidophilus species are normal inhabitants of the small intestine, their bile-resistance can be an important factor in utilizing them as dietary adjuncts for lactose maldigestors.

Bile resistance varies among strains of L. acidophilus (Gilliland et al., 1984). In the present study, the results showed strains 223 and 4356 were the least bile-resistant among 5 strains tested. The other three strains showed no significant differences in bile resistance from each other.

All 5 strains of L. acidophilus tested in this study exhibited significantly more β-galactosidase activity in the presence of oxgall than in its absence. Among them, bile-sensitive strain 4356 showed the highest enzyme activity in both treatments. However, bile resistant strain 107 showed the highest increased enzyme activity when incubated in the presence of oxgall. Meanwhile, strain 223, another bile-sensitive strain, did not show significantly more enzyme activity in the presence of oxgall than did the bile-tolerant strains. Therefore, the increased amount of

enzyme activity in the presence of bile was not related to the degree of bile-resistance. Fisher et al. (1985) and Kim (1981) also reported that enzyme activity increased greatly in the presence of oxgall.

To check the effect of bile on lysis, cells of the 5 strains of L. acidophilus were suspended in buffer and buffer plus 0.3% oxgall. Cell suspensions of all strains under study showed some decrease in turbidity in the presence of oxgall. However, that decrease was also observed for the cells suspended in buffer alone. Since there were no significant differences between decreases of turbidity for cell suspensions in buffer or buffer plus oxgall, the oxgall did not enhance lysis of cells under conditions used in this study. These results combined with the leakage of intracellular material suggest that the increase of enzyme activity in the presence of oxgall was due to increased cellular permeability caused by the oxgall.

The results of the effect of oxgall-pretreatment on cellular retention of β-galactosidase confirmed that cells retained the enzyme inside the cells in the presence of bile. Therefore, it was concluded that bile improved cellular permeability and permitted more substrate to enter the cells and be hydrolyzed. Thus in the small intestine, greater lactose may be permitted to enter the cells and be hydrolyzed due to the presence of bile.

Comparison of specific activities of β-galactosidase for the cell free extracts of the 5 strains showed that

strains 223 and 4356 had the highest specific enzyme activities. Whole cells of strain 4356 also exhibited significantly higher enzyme activity than did cells of the other strains when added in buffer containing oxgall. This suggests that bile does not always enhance β-galactosidase activity of the cultures to the fullest potential.

CHAPTER VI

SUMMARY and CONCLUSIONS

Cells of L. acidophilus have been used for dietary adjuncts to reduce lactose maldigestion, since they are normal inhabitants of small intestine of humans and they contain the lactose hydrolyzing enzyme, β-galactosidase. Because of their residence in the intestine, the influence of bile on L. acidophilus has been of interest.

This study focused on the effect of bile on the cellular integrity and β-galactosidase activity of L. acidophilus. The test for bile-tolerance revealed that significant variation occurred among the strains tested. In addition, the presence of bile significantly increased the β-galactosidase activity for all strains ($P<.05$). However, one bile-sensitive strain, 223, showed no significant difference in enzyme activity from other bile-tolerant ones ($P>.05$), while strain 4356, another bile-sensitive one, showed the highest enzyme activity of all strains in the presence of oxgall. Therefore, it appears that there is no relationship between the degree of bile-tolerance and the increased enzyme activity in the presence of bile.

It was shown that bile (0.3% oxgall) does not significantly enhance lysis of cells of L. acidophilus.

Therefore, it was assumed that the increased enzyme activity was due to either the leakage of the enzyme from the cells or more intake of lactose into the cells through the change of cellular permeability of cell membrane. Our results showed that enzymes were retained inside the cells even after long incubation time in the presence of bile. Thus, the conclusion is that in the presence of bile the permeability of cells of L. acidophilus is increased, permitting more substrate to enter and be hydrolyzed.

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APPENDIX A

DATA FROM TREATMENTS

TABLE 10.

DATA FROM BILE TOLERANCE* OF FIVE STRAINS
OF LACTOBACILLIUS ACIDOPHILUS

Strains		Exp.1	Exp.2	Exp.3	$\bar{Y}$
107	control	4.0	4.0	4.5	4.2
	0.3% oxgall	5.0	4.5	5.0	4.8
223	control	4.0	5.5	5.5	5.0
	0.3% oxgall	6.0	8.0	7.5	7.2
NCFM	control	4.0	4.0	4.0	4.0
	0.3% oxgall	4.0	4.5	5.0	4.5
606	control	4.0	4.0	4.0	4.0
	0.3% oxgall	4.0	4.5	4.5	4.3
4356	control	4.5	5.0	5.0	4.8
	0.3% oxgall	7.0	8.0	8.5	7.8

* Bile tolerance is expressed as hours for A_{620} to increase by 0.3 in PMN broth with and without 0.3% oxgall.

TABLE 11.

DATA FROM EFFECT OF BILE ON β-GALACTOSIDASE ACTIVITY* OF FIVE STRAINS OF LACTOBACILLIUS ACIDOPHILUS

Strains		Exp.1	Exp.2	Exp.3	$\bar{Y}$
107	control	14.5	29.88	18.0	20.79
	0.3% oxgall	40.1	38.28	51.6	43.33
223	control	14.9	34.99	39.2	29.70
	0.3% oxgall	28.1	43.39	42.7	38.06
NCFM	control	13.8	30.98	23.1	22.63
	0.3% oxgall	35.0	47.41	33.2	38.54
606	control	20.7	30.9	24.0	25.20
	0.3% oxgall	30.9	43.39	35.9	36.73
4356	control	33.50	72.98	52.89	53.12
	0.3% oxgall	62.02	72.98	43.94	59.65

* β-galactosidase activity is expressed as microgram of ONP liberated during 10 min. incubation.

TABLE 12.

DATA FROM EFFECT OF BILE ON CELLULAR INTEGRITY
OF L. ACIDOPHILUS 107

		A_{620nm}				
		0 min	10 min	20 min	30 min	40 min
control	exp.1	0.523	0.432	0.432	0.420	0.415
	exp.2	0.523	0.469	0.469	0.456	0.444
	exp.3	0.509	0.450	0.444	0.432	0.420
	$\bar{Y}$	0.518	0.450	0.448	0.436	0.426
0.3% oxgall	exp.1	0.444	0.398	0.387	0.387	0.377
	exp.2	0.495	0.456	0.444	0.438	0.420
	exp.3	0.469	0.432	0.415	0.409	0.398
	$\bar{Y}$	0.469	0.429	0.415	0.411	0.398

TABLE 13.

DATA FROM EFFECT OF BILE ON CELLULAR INTEGRITY OF L. ACIDOPHILUS 223

		A_{620nm}				
		0 min	10 min	20 min	30 min	40 min
control	exp.1	0.585	0.456	0.450	0.444	0.444
	exp.2	0.509	0.469	0.462	0.456	0.450
	exp.3	0.561	0.481	0.475	0.469	0.456
	$\bar{Y}$	0.552	0.469	0.462	0.456	0.450
0.3% oxgall	exp.1	0.469	0.432	0.420	0.415	0.409
	exp.2	0.469	0.420	0.420	0.409	0.398
	exp.3	0.481	0.444	0.432	0.420	0.420
	$\bar{Y}$	0.473	0.432	0.424	0.415	0.409

TABLE 14.

DATA FROM EFFECT OF BILE ON CELLULAR INTEGRITY
OF L. ACIDOPHILUS NCFM

		A_{620nm}				
		0 min	10 min	20 min	30 min	40 min
control	exp.1	0.553	0.469	0.469	0.456	0.450
	exp.2	0.438	0.398	0.398	0.387	0.382
	exp.3	0.495	0.432	0.432	0.420	0.409
	$\bar{Y}$	0.495	0.433	0.433	0.421	0.414
0.3% oxgall	exp.1	0.481	0.444	0.432	0.409	0.409
	exp.2	0.415	0.387	0.367	0.357	0.347
	exp.3	0.432	0.409	0.398	0.387	0.377
	$\bar{Y}$	0.443	0.413	0.399	0.384	0.378

TABLE 15.

DATA FROM EFFECT OF BILE ON CELLULAR INTEGRITY
OF L. ACIDOPHILUS 606

		A_{620nm}				
		0 min	10 min	20 min	30 min	40 min
control	exp.1	0.509	0.481	0.469	0.456	0.456
	exp.2	0.509	0.481	0.475	0.469	0.469
	exp.3	0.538	0.456	0.444	0.444	0.420
	$\bar{Y}$	0.519	0.473	0.463	0.456	0.448
0.3% oxgall	exp.1	0.469	0.426	0.409	0.398	0.387
	exp.2	0.481	0.444	0.420	0.409	0.398
	exp.3	0.456	0.409	0.398	0.387	0.377
	$\bar{Y}$	0.469	0.426	0.409	0.398	0.387

TABLE 16.

DATA FROM EFFECT OF BILE ON CELLULAR INTEGRITY
OF L. ACIDOPHILUS 4356

		A_{620nm}				
		0 min	10 min	20 min	30 min	40 min
control	exp.1	0.538	0.488	0.456	0.456	0.456
	exp.2	0.481	0.444	0.438	0.432	0.432
	exp.3	0.530	0.469	0.462	0.444	0.444
	$\bar{Y}$	0.516	0.467	0.452	0.444	0.444
0.3% oxgall	exp.1	0.488	0.456	0.444	0.438	0.438
	exp.2	0.444	0.409	0.409	0.409	0.392
	exp.3	0.469	0.444	0.420	0.420	0.420
	$\bar{Y}$	0.467	0.436	0.424	0.422	0.417

TABLE 17.

DATA FROM COMPARISON OF SPECIFIC ACTIVITIES* OF FIVE STRAINS OF L. ACIDOPHILUS

	107	223	NCFM	606	4356
exp.1	0.80	2.80	0.89	1.34	2.49
exp.2	1.18	2.41	0.98	1.07	2.05
exp.3	0.27	2.08	0.26	0.44	1.68
$\bar{Y}$	0.75	2.43	0.71	0.95	2.07

* Specific activity is expressed as micromoles of ONP liberated per mg of protein per minute at 37^0C.

TABLE 18.

DATA FROM EFFECT OF BILE ON CELLULAR RETENTION* OF β-GALACTOSIDASE OF LACTOBACILLUS ACIDOPHILUS

Supernatant	Strains	Exp.1	Exp.2	Exp.3	$\bar{Y}$
Control	107	0.000	0.000	0.219	0.073
	223	0.000	0.000	0.365	0.122
	NCFM	0.000	0.000	0.256	0.085
	606	0.000	0.000	0.511	0.170
	4356	1.860	1.205	0.183	1.083
oxgall-pretreated	107	0.000	0.000	0.000	0.000
	223	1.352	1.278	0.329	0.986
	NCFM	0.000	0.000	0.110	0.037
	606	0.000	0.000	0.000	0.000
	4356	3.760	3.726	0.000	2.495

* Cellular retention is expressed as β-galactosidase activity(=microgram of ONP liberated per 10 min. incubation)

TABLE 19.

DATA FROM EFFECT OF BILE ON CELLULAR RETENTION* OF β-GALACTOSIDASE OF LACTOBACILLUS ACIDOPHILUS

Cells	Strains	Exp.1	Exp.2	Exp.3	$\bar{Y}$
Control	107	7.34	6.50	2.19	5.34
	223	33.46	42.01	6.06	27.18
	NCFM	4.27	6.36	3.00	4.54
	606	8.40	3.43	2.30	4.71
	4356	22.90	41.20	19.84	27.98
oxgall-pretreated	107	15.16	4.27	2.19	7.21
	223	110.93	102.75	23.89	79.19
	NCFM	6.21	5.30	2.52	4.68
	606	20.53	14.03	5.66	13.41
	4356	32.29	53.73	57.68	47.90

* Cellular retention is expressed as β-galactosidase activity(=microgram of ONP liberated per 10 min. incubation)

TABLE 20.

DATA FROM EFFECT OF BILE ON LEAKAGE OF INTRACELLULAR MATERIALS FROM CELLS OF L. ACIDOPHILUS 107

		A_{260nm}			
		0 min	30 min	60 min	90 min
control	exp.1	0.058	0.049	0.075	0.123
	exp.2	0.065	0.062	0.094	0.180
	exp.3	0.100	0.055	0.099	0.118
	$\bar{Y}$	0.074	0.055	0.089	0.140
oxgall-treated	exp.1	0.079	0.087	0.143	0.216
	exp.2	0.056	0.089	0.187	0.236
	exp.3	0.116	0.111	0.208	0.225
	$\bar{Y}$	0.084	0.096	0.179	0.226

TABLE 21.

DATA FROM EFFECT OF BILE ON LEAKAGE OF INTRACELLULAR MATERIALS FROM CELLS OF L. ACIDOPHILUS 223

		A_{260nm}			
		0 min	30 min	60 min	90 min
control	exp.1	0.063	0.078	0.103	0.110
	exp.2	0.085	0.087	0.096	0.115
	exp.3	0.118	0.092	0.115	0.100
	$\bar{Y}$	0.089	0.086	0.105	0.108
oxgall-treated	exp.1	0.062	0.174	0.280	0.364
	exp.2	0.060	0.127	0.196	0.283
	exp.3	0.063	0.116	0.194	0.276
	$\bar{Y}$	0.062	0.139	0.223	0.308

TABLE 22.

DATA FROM EFFECT OF BILE ON LEAKAGE OF INTRACELLULAR MATERIALS FROM CELLS OF L. ACIDOPHILUS NCFM

		A_{260nm}			
		0 min	30 min	60 min	90 min
control	exp.1	0.090	0.061	0.082	0.100
	exp.2	0.073	0.078	0.108	0.151
	exp.3	0,123	0.067	0.110	0.116
	$\bar{Y}$	0.095	0.069	0.100	0.122
oxgall-treated	exp.1	0.052	0.069	0.164	0.220
	exp.2	0.021	0.070	0.170	0.193
	exp.3	0.079	0.140	0.245	0.266
	$\bar{Y}$	0.051	0.093	0.193	0.226

TABLE 23.

DATA FROM EFFECT OF BILE ON LEAKAGE OF INTRACELLULAR MATERIALS FROM CELLS OF L. ACIDOPHILUS 606

		A_{260nm}			
		0 min	30 min	60 min	90 min
control	exp.1	0.162	0.063	0.076	0.111
	exp.2	0.126	0.099	0.094	0.092
	exp.3	0.093	0.065	0.106	0.059
	$\bar{Y}$	0.127	0.076	0.092	0.087
oxgall-treated	exp.1	0.059	0.146	0.216	0.266
	exp.2	0.024	0.106	0.241	0.265
	exp.3	0.094	0.175	0.249	0.272
	$\bar{Y}$	0.059	0.142	0.235	0.268

TABLE 24.

DATA FROM EFFECT OF BILE ON LEAKAGE OF INTRACELLULAR MATERIALS FROM CELLS OF L. ACIDOPHILUS 4356

		A_{260nm}			
		0 min	30 min	60 min	90 min
control	exp.1	0.118	0.166	0.224	0.264
	exp.2	0.144	0.195	0.204	0.231
	exp.3	0.136	0.148	0.162	0.194
	$\bar{Y}$	0.133	0.170	0.197	0.230
oxgall-treated	exp.1	0.000	0.379	0.445	0.428
	exp.2	0.098	0.399	0.437	0.501
	exp.3	0.150	0.392	0.449	0.466
	$\bar{Y}$	0.083	0.390	0.444	0.365

APPENDIX B

STATISTICAL ANALYSES

TABLE 25.

ANALYSIS OF VARIANCE TABLE* - BILE TOLERANCE OF DIFFERENT STRAINS OF LACTOBACILLUS ACIDOPHILUS

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	11	51.0833	4.6439	25.46
Error	18	3.2833	0.1824	
Total	29	54.3666		

*Data from Table 10
OSL <.05
$LSD_{.05}$ = 0.7327

TABLE 26.

ANALYSIS OF VARIANCE TABLE* - EFFECT OF BILE ON β-GALACTOSIDASE ACTIVITY OF LACTOBACILLUS ACIDOPHILUS

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	11	5584.8162	507.7106	7.75
Error	18	1179.5085	65.5283	
Total	29	6764.3247		

*Data from Table 11
OSL <.05
$LSD_{.05}$ = 6.210

TABLE 27.

ANALYSIS OF VARIANCE TABLE* - EFFECT OF BILE ON CELLULAR INTEGRITY OF LACTOBACILLUS ACIDOPHILUS 107

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	11	0.0400	0.0036	44.02
Error	18	0.0015	0.0001	
Total	29	0.0415		

*Data from Table 12
OSL<.05
$LSD_{.05}$ = 0.0060

TABLE 28.

ANALYSIS OF VARIANCE TABLE* - EFFECT OF BILE ON CELLULAR INTEGRITY OF LACTOBACILLUS ACIDOPHILUS 223

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	11	0.0471	0.0043	24.10
Error	18	0.0032	0.0002	
Total	29	0.0503		

*Data from Table 13
OSL<.05
$LSD_{.05}$ =0.0109

TABLE 29.

ANALYSIS OF VARIANCE TABLE* - EFFECT OF BILE ON CELLULAR INTEGRITY OF LACTOBACILLUS ACIDOPHILUS NCFM

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	11	0.0561	0.0051	33.65
Error	18	0.0027	0.0002	
Total	29	0.0588		

*Data from Table 14
OSL <.05
$LSD_{.05}$ =0.0108

TABLE 30.

ANALYSIS OF VARIANCE TABLE* - EFFECT OF BILE ON CELLULAR INTEGRITY OF LACTOBACILLUS ACIDOPHILUS 606

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	11	0.0457	0.0042	34.42
Error	18	0.0022	0.0001	
Total	29	0.0479		

*Data from Table 15
OSL <.05
$LSD_{.05}$ =0.0077

TABLE 31.

ANALYSIS OF VARIANCE TABLE* - EFFECT OF BILE ON CELLULAR INTEGRITY OF LACTOBACILLUS ACIDOPHILUS 4356

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	11	0.0302	0.0027	42.91
Error	18	0.0012	0.0001	
Total	29	0.0314		

*Data from Table 16
OSL <.05
$LSD_{.05}$ =0.0077

TABLE 32.

ANALYSIS OF VARIANCE TABLE* - COMPARISON OF SPECIFIC ACTIVITIES OF 5 STRAINS OF LACTOBACILLUS ACIDOPHILUS

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	4	7.8416	1.9604	11.26
Error	10	1.7409	0.1741	
Total	14	9.5825		

*Data from Table 17
OSL <.05
$LSD_{.05}$ =0.7591

TABLE 33.

ANALYSIS OF VARIANCE TABLE* - EFFECT OF BILE ON CELLULAR RETENTION OF β-GALACTOSIDASE OF LACTOBACILLUS ACIDOPHILUS (IN CELL FRACTION)

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	11	17854.5525	1623.1411	6.08
Error	18	4801.8276	266.7682	
Total	29	22656.3801		

*Data from Table 19
OSL <.05
$LSD_{.05}$ =12.53

VITA

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