EFFECT OF FEEDING UNFERMENTED MILK CONTAINING *LACTOBACILLUS ACIDOPHILUS* ON THE INTESTINAL FLORA OF DAIRY CALVES

By

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CHAPTER I

INTRODUCTION

Elie Metcknikoff is generally credited with the initial idea of ingesting Lactobacillus acidophilus as a therapeutic treatment of gastrointestinal disorders (Metchnikoff, 1908). He suggested that putrefactive organisms in the intestinal tract released and caused illnesses that were associated with old age. Ingestion of L. acidophilus relieved the patients suffering from these illnesses and changed the intestinal flora to a fermentative type.

The usual mode of administration of L. acidophilus has been via the ingestion of milk fermented with the organism. The fermented product has been characterized as having a very unpleasant acid flavor and has not been accepted for routine consumption. The acceptance was improved by the development of an unfermented milk containing a concentrated culture of L. acidophilus. The flavor of this product is the same as that of fresh pasteurized milk.

The enteric flora of both human and animals represents a diverse collection of microorganisms. The numbers and types of microorganisms inherent with each animal is controlled by various mechanisms. Many studies indicate that L. acidophilus exhibits in vitro and in vivo antagonisms toward enteric pathogenic bacteria which helps to establish a normal balance of the intestinal flora. Some researchers have suggested that host specificity exists among strains of L. acidophilus

with respect to the animal species in which they will establish.

In this thesis research, unfermented milk containing cells of L. acidophilus from two different animal species was ingested by neonatal calves to determine the possibility of host specificity of the organism. Another objective was to determine how the ingestion of two strains of L. acidophilus affected the numbers of coliform bacteria appearing in the intestinal flora of the calves.

CHAPTER II

REVIEW OF LITERATURE

Cultured Milk Therapy

In the early 1900's Elie Metchnikoff initiated the concept of consuming cultured milk products for therapeutic purposes. Metchnikoff (1908) theorized that illnesses associated with old age were caused by metabolic end products formed by putrefactive microorganisms in the intestinal tract. He advocated that including fermented milk containing Bacillus bulgaricus in the diet would transform the intestinal flora to a more aciduric type, producing few or no detectable toxic metabolites. Insufficient evidence prevented any strong conclusions concerning the establishment of Bacillus bulgaricus in the intestinal tract (Kopeloff, 1926). However, when massive amounts of milk fermented with Lactobacillus acidophilus were consumed for several days or weeks the organism was established as the predominant bacterium in the intestinal tract.

Rahe (1915) reported B. acidophilus rather than B. bulgaricus could be established in the intestinal tract. In 1920 Holland changed the name of B. acidophilus to L. acidophilus. The difficulty in distinguishing between B. bulgaricus and L. acidophilus was due to inadequate identification techniques (Wheater, 1955). The organism identified by Metchnikoff presumably was L. acidophilus since B. bulgaricus does not survive passage through the intestinal tract (Frost et al., 1931). Several researchers reported that L. acidophilus is a

normal inhabitant of the human intestinal tract (Haenal, 1970; Gilliland et al., 1975; Moore et al., 1975).

Kopeloff (1926) indicated that patients suffering from diarrhea were relieved of symptoms by the ingestion of L. acidophilus. Rettger et al. (1935) suggested feeding L. acidophilus in the therapeutic treatment of patients suffering from constipation. Toxic amines that are produced by putrefactive bacteria in the lower intestinal tract may be absorbed into the blood of certain individuals and induce a condition called encephalopathy. Mac Beth et al. (1965) reported that encephalopathy could be treated effectively by the ingestion of L. acidophilus. The organisms that produced toxic amines were suppressed by L. acidophilus. Shaw and Muth (1937) successfully treated scours in calves by feeding them milk fermented with L. acidophilus.

Development of an Unfermented Milk Product

Myers (1931) observed that the bacterial flora in the feces of rats and humans was changed to a predominance of lactobacilli when they were fed an unfermented milk containing L. acidophilus. The milk contained a high concentration of viable organisms similar to the fermented product, except the viable counts in the unfermented milk were maintained longer than in the fermented product. He reported that the unfermented milk tasted better and was more appealing for human consumption. Kulp (1931) claimed the product maintains palatability when stored at $5^{o}C$ for 2 days to 1 week. The viability of L. acidophilus in the product was retained when stored at the same temperature.

Development of procedures for producing frozen concentrated cultures has made the commercial production of such an unfermented milk product

feasible. The culture can be grown under closely controlled conditions in a liquid medium that provides all of the necessary nutrients for optimum growth (Gilliland and Speck, 1974). The cells of L. acidophilus can then be harvested by centrifugation and resuspended in a smaller volume of milk. The resulting concentrated culture can then be frozen in liquid nitrogen and shipped to dairy processing plants. The frozen concentrated culture is thawed and the desired amount added to cold pasteurized, low fat milk, packaged and immediately refrigerated (Gilliland et al., 1978). The resulting milk beverage having the same flavor as freshly pasteurized milk contains a large number of viable cells of L. acidophilus.

Establishment of Lactobacillus acidophilus in the Intestinal Tract

Many efforts indicated that a predominance of lactobacilli may be established in the intestinal tract by feeding products containing L. acidophilus to both humans and animals. Stark et al. (1934) showed that consumption of milk cultured with L. acidophilus resulted in an increase in the number of lactobacilli occurring in the feces. This study included 124 persons who consumed the product for 18 months. They reported the number of lactobacilli in the feces diminished 2 to 6 weeks after the subjects stopped consuming the milk. The establishment of L. acidophilus in the intestinal tract, as noted by Hawley et al (1959), depends on the number of viable cells of L. acidophilus fed to the subject. Speck (1976) indicated daily ingestion of 1×10^8 to 1×10^9 viable L. acidophilus is needed by humans to replace lactobacilli lost in oral antibiotic therapy and to help recover a

balance in the normal intestinal flora. Gilliland et al. (1978) observed an increase in the number of fecal lactobacilli in healthy males after consuming unfermented milk containing L. acidophilus even when it contained low levels (5×10^6/ml) of the organism. They suggested that the increase in fecal lactobacilli was associated with a shift in type(s) of lactobacilli in the intestinal tract. The number of fecal lactobacilli decreased after the subjects stopped consuming the milk. Frost et al. (1931) reported that the consumption of lactose and dextrin may stimulate the growth of L. acidophilus in the intestinal tract. However, they noted that the ingestion of a supplemental carbohydrate alone without L. acidophilus would not establish a predominance of lactobacilli in the intestinal tract.

Paul and Hoskins (1972) were unable to demonstrate any change in the number of intestinal lactobacilli due to feeding a lyophilized preparation of L. acidophilus to humans. Similar results were obtained by Conn and Flock (1970) who fed a dried capsule preparation of L. acidophilus to normal and cirrhotic patients. Unsuccessful attempts to establish lactobacilli or increase their numbers in the intestinal tract may have been the result of feeding cell preparations containing a lactobacillus other than L. acidophilus or a cell preparation with a low viable count (Gilliland and Speck, 1977a).

Development of the Intestinal Flora in Humans and Animals

Results from several studies have shown that the development of the intestinal tract in the young, interactions among the intestinal microorganisms, and environment of the host influence the establishment of

the intestinal flora (Smith and Crabb, 1961; Donaldson, 1964). Smith and Crabb (1961) observed that the resemblance in the fecal flora between young animals of different species was associated with a similarity of the initial diet, mainly milk. They reported the total viable count of the fecal bacteria in all animal species reached a peak in early stages of life; a subsequent decrease in counts and a change of microbial types was inherent with age. They indicated the development and successive changes in the fecal flora following birth is unique for each animal species.

Gorbach et al. (1967) have shown variations in the intestinal microflora between individuals for both humans and animals. They indicated that the small intestine contains lactobacilli, streptococci, fungi, and staphylococci. The microorganisms occurring in most numerous amounts in the colon include: bacteroides, coliforms, and anaerobic lactobacilli.

Donaldson (1964) indicated that the composition of the intestinal flora depends on the environment of the host animal. When Dubos et al. (1962) maintained mice in an unusually clean environment the fecal flora was distinctly different from the flora in the feces collected from mice exposed to normal conditions.

Savage (1972) showed that certain microbial types are associated intimately with the mucosal epithelium in certain regions of the gastrointestinal tract of mice. The microbial mucosal association was shown to affect the physiological conditions of the intestinal tract. After feeding a species of Lactobacillus to germ-free mice, Savage (1972) observed a depression of alkaline phosphatase activity in the epithelial cells of the duodenal microvilli. The enzyme was depressed

less rapidly when Bacteriodes was fed to germ-free mice. He concluded that only species of Lactobacillus not Bacteroides, are intimately associated with the mucosal epithelia of the duodenum. His results showed microorganisms play a role in a normal function of the intestinal tract.

Bryant (1972) and Donaldson (1964) claimed the microbial types present in the intestinal lumen are determined partly by interactions between bacteria species. Any antibiotics or inhibitory substances produced by one microbial type may effect the balance of microorganisms present in the intestinal tract. L. acidophilus has been shown to be antagonistic in vitro against such enteropathogenic organisms as Staphylococcus aureus, Clostridium perfringens, Salmonella typhimurium, and Escherichia coli (Gilliland, 1979). Mikolajcik and Hamdan (1975) and Sandine (1979) reported that the antagonism is partly due to the production of antibiotic-like substances as acidophilin, acidolin, or lactocidin. The antagonistic action produced by L. acidophilus may be due to a combination of substances that include acids, hydrogen peroxide, and antibiotics (Gilliland, 1979). Sabine (1963) observed a zone of inhibition of Staphylococcus aureus surrounding an area where L. acidophilus grew on the same plate. He reported that the zone was not due to acid production since there was no pH gradient on the plate. Vincent et al. (1959) obtained several strains of L. acidophilus from animals that produced lactocidin. Lactocidin had a broad antibacterial spectrum and he suggested that L. acidophilus helps maintain a normal population of enteric bacteria from the production of lactocidin in the intestinal tract. Hosono et al. (1977) isolated a peptide-like antibiotic substance from a strain of L. acidophilus that was different

from lactocidin and acidolin. Ellinger et al. (1978) showed that feeding milk containing L. acidophilus (from an unknown source) to young dairy calves resulted in fewer coliform bacteria in their feces than after feeding whole milk.

Host Specificity of Lactobacillus acidophilus

L. acidophilus may manifest a certain amount of host specificity. In identifying L. acidophilus isolated from fresh feces of humans, swine, and chickens, Mitusoka (1968) encountered difficulties in classifying several of the animal strains. Several biotypes were established for the unidentified strains of L. acidophilus. Gilliland et al. (1975) found minor differences in the biochemical fermentation patterns and melting points of deoxyribonucleic acids of L. acidophilus isolated from humans, pigs, and chickens. Shimohashi and Mutai (1976) observed specific antigens associated with several biotypes of L. acidophilus isolated from human and animal sources. A strain of L. acidophilus isolated from a human failed to be established in the intestinal tract of a germ-free chicken (Morishita et al., 1971). However, a strain of L. acidophilus isolated from a conventional chicken was easily established in the intestinal tract of the germ-free chicken. In view of the evidence given on strains of L. acidophilus with different biotypes and problems related to establishing different biotypes in certain animals, it appears that a host-specific relationship may exist among strains of L. acidophilus

CHAPTER III

EXPERIMENTAL PROCEDURE

Source and Maintenance of Cultures

L. acidophilus NCFM, originally isolated from human intestines was obtained from the Food Science Department of North Carolina State University. L. acidophilus C-28 was isolated from the ileum of a young calf. Each culture was routinely propagated in sterile 10% nonfat milk solids (NFMS) using a 1% inoculum and incubation at 37°C for 18 hours.

Enumeration of Bacteria

Enumeration of Facultative Lactobacilli

The necessary decimal dilutions of the samples were prepared in sterile 99 ml dilution blanks. The dilution blanks contained 0.1% peptone (Difco Laboratories, Inc., Detroit, Michigan) and 0.01% silicone antifoamer (Sigma Chemical Company, St. Louis, Missouri) in distilled water. They were sterilized by heating at 121°C for 15 minutes. The procedures for making the dilutions were in accordance with those in Standard Methods for the Examination of Dairy Products (Marth, 1978).

Duplicate plates of the required dilutions were poured with Lactobacillus Selection (LBS) Agar (Baltimore Biological Laboratories, Cockeysville, Maryland), a medium selective for lactobacilli (Rogosa

et al., 1951). A second set of duplicate plates was poured with LBS agar containing 0.1% oxgall (Baltimore Biological Laboratories, Cockeysville, Maryland), designated as LBSO agar, to enumerate bile resistant lactobacilli (Gilliland et al., 1975). The plates were placed in plastic bags, flushed with carbon dioxide for 1 minute, sealed and incubated for 72 hours at 37°C (Gilliland et al., 1975). The colonies were counted with the aid of a Quebec Colony Counter.

The predominating types of colonies observed on LBS agar were isolated for identification. Cells from five colonies from each sample were transferred with a sterile needle into a tube containing 10 ml of sterile lactobacilli MRS broth (Difco Laboratory, Inc., Detroit, Michigan) and incubated at 37°C until growth was evidenced (turbidity). The isolates were then inoculated via flame sterilized needles (stabbed) into screw capped tubes containing approximately 10 ml of MRS agar. The stab cultures were incubated 18 hours at 37°C and stored at 5°C. The MRS agar was prepared by adding 1.5% agar to lactobacilli MRS broth prior to autoclaving at 121°C for 15 minutes. The stab cultures were subcultured into fresh tubes of MRS agar at monthly intervals until their identities were determined.

Enumeration of Coliform Bacteria

Dilutions were prepared in the same manner as described in the previous section. Duplicate plates of the proper dilution were poured with Violet Red Bile Agar (Difco Laboratories, Inc., Detroit, Michigan). After solidifying, an overlay (about 5 ml) of Violet Red Bile Agar was poured onto each plate. The plates were incubated at 37°C for 24 hours. Typical coliform colonies were counted in accordance to the procedures

in Standard Methods for the Examination of Dairy Products (Marth, 1978).

Identification of Facultative Lactobacilli

Each culture was streaked onto duplicate plates of MRS agar and incubated anaerobically in a Gas Pak System (Baltimore Biological Laboratories, Cockeysville, Maryland) at 37°C. This provided an opportunity to observe colonial morphology of surface colonies and to determine if only one colony type was present for each culture. One plate was used for obtaining a sample for preparing a Gram stain. The Burke method (Burke, 1922) was used. After preparing the slides for staining, the plate was flooded with 3% H_2O_2 to test for the presence of catalase in the culture. Effervescence from the colonies was considered a positive test for catalase. For this study, catalase negative gram positive rods were assumed to be lactobacilli.

The second streak plate for each culture was utilized as a source of inocula for determining the ability of the organism to ferment or utilize certain substrates. The Minitek System (Baltimore Biological Laboratories, Cockeysville, Maryland) was utilized for this as described by Gilliland and Speck (1977b). The cultures were evaluated for the following: ammonia production from arginine, esculin hydrolysis, and fermentation of amygdalin, arabinose, cellobiose, galactose, glucose, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhammose, salicin, sorbitol, sucrose, trehalose, and xylose. The ability of the culture to grow at 15°C was also checked. The culture was inoculated into a sterile screw cap tube containing 10 ml of sterile lactobacilli MRS broth, using an inoculation loop (0.01 ml). The tubes were incubated at 15°C for 1 week. Visual turbidity was taken as an indication of positive growth.

Preparation of Milk Containing *Lactobacillus acidophilus*

Cells of *L. acidophilus* were propagated in lactobacillus MRS broth. The inoculum for the broth was prepared by inoculating (1%) 10% NFMS (which had been autoclaved 15 minutes at 121°C) with *L. acidophilus* C-28 and incubating it 18 hours at 37°C. Ten ml of the resulting milk culture was aseptically inoculated into 1000 ml of lactobacilli MRS broth and incubated at 37°C for 18 hours. The broth culture was chilled in an ice bath. The cells were harvested by centrifuging the culture in a Sorvall RC-5 Superspeed Refrigerated Centrifuge (Du Pont Company, Newton, Conneticut) at 10,444 xg, for 20 minutes at 5°C. The supernatant fluid was discarded and the pellets of cells were scraped into a cold sterile blender cup with the aid of a sterile spatula. The cells were resuspended in 100 ml of cold pasteurized whole milk using a Waring Blender (Elerbach Corporation, Ann Arbor, Michigan) set at high speed for 1 minute. A slow stream of carbon dioxide was introduced into the head space over the suspension during the blending to help minimize the amount of oxygen which might be incorporated into the mixture. The final volume of the cell suspension was 120 ml. A quantity of 40 ml was added to each of three 6 gallon containers of cold pasteurized whole milk. The cells were suspended in the milk by vigorously shaking each container.

Milk containing *L. acidophilus* NCFM was prepared in the same manner as milk containing *L. acidophilus* C-28 with the exception that twice as much broth or 2000 ml was utilized for growing the cells. This was necessary because preliminary experiments showed that the number of cells of *L. acidophilus* C-28 produced in the broth was twice that

produced by L. acidophilus NCFM.

Immediately following preparation, the milk containing L. acidophilus was checked for numbers of facultative lactobacilli.

The milk was transferred to the experimental area where it was stored at 5°C. Several containers of milk without cells of L. acidophilus were also taken to the experimental area. The milk was prepared weekly and milk remaining after 1 week was discarded.

Prior to conducting feeding trials, the stability of L. acidophilus in refrigerated whole milk was determined. The prepared milk was assayed by plating on LBS agar and LBSO agar on days 1, 3, 5, and 7 during storage at 5°C.

Collection and Examination of Colostrum

Five lots of colostrum were collected from several post partum cows. Each lot was thoroughly mixed and immediately dispensed in half pint milk cartons (Excello Corporation, Detroit, Michigan) in the University dairy processing plant. The packaged colostrum was frozen at -10°C and held there until needed.

One carton from each lot of colostrum was thawed in warm water (45°C). One ml was aseptically removed from each carton after mixing and placed in a 99 ml dilution blank to be checked for coliform bacteria. The lots of colostrum containing greater than 1×10^4 coliforms per ml were discarded.

Feeding Trial I

Collection and Assignment of Calves to Treatment Groups

Neonatal Holstein and Ayrshire bull calves were selected as test

animals for the feeding trial. The calves were allowed to remain with the cow 2 to 4 hours post partum in order to receive a first feeding of colostrum. The naval cords of the calves were swabbed with iodine and covered to help prevent infective contamination while transporting the calves to the experimental area. Care was exercised to minimize stress to the animals while being moved. Five calves were randomly assigned to each treatment group (Steele and Torre, 1960). The calves were placed in individual pre-cleaned stalls so that no two calves of the same treatment were adjacent. The stalls had been sanitized with hot water ($55^{o}C$) containing Roccal-D Disinfectant (Winthrop Laboratories, New York). The calves remained on the feeding trial for 14 days.

Feeding Procedures

All calves were fed colostrum from a frozen supply for the second feeding. Prior to feeding, the colostrum was thawed and warmed to $25^{o}C$ in a clean stainless steel container that was immersed in hot water ($55^{o}C$). Colostrum from each of 3 lots was thawed and fed as a uniform pool to each calf. The colostrum pool was formulated in the following manner: the appropriate number of cartons of frozen colostrum were thawed representing 1 part of lot number 3, 2 parts of lot number 4, and 1 part of lot number 5. Each calf was fed 12 1/2% of its metabolic size ($W^{.75}$) of colostrum 10 to 14 hours following birth.

Feeding of the experimental milks was started with the third feeding. The calves received 10% of their metabolic size ($W^{.75}$) of milk 8 hours following the feeding of colostrum. The same amount of milk was given to each calf twice daily, at 7:00 a.m. and 5:00 p.m. during the 14 days of the experiment. The calves were fed using a

calf nursing bottle (Albers, Carnation, Washington). The bottles were sanitized with hot water (55°C) containing Roccal-D disinfectant and thoroughly rinsed prior to use for each feeding. The amount of colostrum or milk to be fed to each calf was measured in the nursing bottle using Toledo Tabletop Scales (Toledo, Ohio).

Analysis of Feces

Sampling and Preparation of Samples for Analysis. Fecal samples were obtained from each calf on days 1, 7, and 14. The first sample was taken immediately following the initial milk feeding. The samples were obtained by massaging the calf's rectum with the forefinger. A disposable surgical glove was worn for this procedure. The sample was collected in a clean unused 8 oz. cottage cheese carton. The carton was promptly taken to the laboratory and stored in a refrigerator at 5°C. The sample was maintained in the refrigerator no longer than 2-3 hours prior to laboratory analysis.

Each sample was mixed thoroughly using a sterile tongue depressor. Six grams of feces were weighed into an empty sterile dilution bottle containing 20-25 glass beads (1/8 inch in diameter). Twenty-four ml of sterile peptone dilution water (0.1% peptone and 0.01% antifoamer) was added aseptically to the bottle. (For samples weighing less than 6 grams, the amount of dilution water added to feces was equal to 4 times the weight of the sample.) The bottle containing the fecal sample was shaken in a 1 foot arc for 3 minutes. The undissolved solids were allowed to settle to the bottom of the bottle. The entire sample was then filtered through 4 layers of sterile cheese cloth into an empty sterile dilution bottle and immediately placed in an ice bath.

Microbiological Examination. The fecal sample filtrates were examined for number of lactobacilli and coliforms using plating procedures. The predominating types of colonies (lactobacilli) observed on LBS agar (5/sample) for the samples collected at day 7 were isolated for identification.

Solids Determination. The fecal sample filtrate was mixed by swirling the bottle and 2.5 ml was weighed into each of 4 predried and tared aluminum weighing pans. The pan plus sample was weighed to 0.1 mg on a Mettler Analytical Balance. The samples were dried in a convection air oven at 100^{o}C for 16 hours. Each pan containing the dried sample was placed in a dessicator to cool. After weighing the pans containing the dried sample, the grams of dry matter per ml of sample filtrate was calculated. This was used as a basis for computing bacterial counts per gram dry weight.

Feeding Trial II

All procedures were the same for the second feeding trial as in Feeding Trial I with the exception that four calves were assigned to each treatment group. Each calf was administered 5cc of Reo-vac oral antiviral vaccine (Norden Laboratories, Lincoln, Nebraska) to prevent viral induced scours. Fecal samples were collected and analyzed on days 1 and 7. Also, at 10 days after birth the animals were sacrificed in order to surgically remove the small and large intestines for microbiological analyses. All calves were sacrificed 2 hours after being fed in order to help assure that similar amounts of milk remained in the intestines.

Surgical Procedures

The calves were immobilized with a large dose of 2% Xylocaine (Astra, Worcester, Massachusetts) injected into the jugular vein. The calf was placed in a recumbent position on the floor of the surgery area. The jugular vein was severed to drain the blood from the calf. The abdomen was shaven and cleaned with iodine. A 6 1/2 inch long incision was made through the abdominal wall without penetrating any underlying intestines. The intestines were pulled from the abdominal cavity and placed onto sterile surgical cloth. A 4 to 6 inch segment from the central portion of both the small and large intestines were removed from the intestinal tract, immediately placed in a sterile Whirl Pak bag (Curtin Matheson Scientific, Inc., Houston, Texas), and set in an ice bath. The samples were immediately taken to the laboratory for analyses.

Microbiological Examination of the Intestinal Segments

Each segment was weighed into a cold sterile blender cup shortly after surgical removal. Peptone dilution water was added to the cup in an amount equal to 4 times the weight of the segment. The samples were blended on a Waring Blender at high speed for 1 minute. The blended sample was filtered through a cheese cloth in the same manner as used for the fecal samples, described in Feeding Trial I. The filtrate was then assayed for the total solids, numbers of coliforms and lactobacilli.

Statistical Analyses

An analysis of variance was used to determine if the day of sampling and/or treatment had a significant effect upon the numbers of lactobacilli and coliform bacteria in the feces or intestines from the calves. An analysis of variance was also used to determine if feeding L. acidophilus influenced the occurrence of L. acidophilus in feces of 7 day old calves. Least significant differences were used to compare the means if significant variation was indicated. The methods for these analyses are outlined in Principles and Procedures of Statistics (Steele and Torre, 1960).

CHAPTER IV

RESULTS

Confirmation of Identity of Cultures of *Lactobacillus acidophilus* Used in Feeding Trials

To confirm the identity of the lactobacilli as strains of *Lactobacillus acidophilus*, a series of tests were performed using the Minitek system. The characteristics of both strains matched those of *L. acidophilus* as described in *Bergey's Manual of Determinative Bacteriology* 8th Edition (Buchanan, 1974) with the exception that *L. acidophilus* C-28 did not ferment trehalose (Table I).

Stability of *Lactobacillus acidophilus* in Milk

There was no appreciable decrease in the numbers of viable lactobacilli and their bile resistance (counts on LBS and LBSO) in the milk containing *L. acidophilus* C-28 stored at $5^{o}C$ for 7 days (Table II). Results from studies at North Carolina State University (Austin, 1977) indicated that milk containing *L. acidophilus* NCFM could be stored at $5^{o}C$ for 1 week with no decrease in numbers of viable lactobacilli. There was some variation in the numbers of lactobacilli in the milk prepared at weekly intervals during both feeding trials (Table III). The numbers of lactobacilli (counts on LBS) in milk containing *L. acidophilus* C-28 were as high as 2.0×10^{7}/ml

TABLE I

CONFIRMATION OF IDENTITY OF *LACTOBACILLUS ACIDOPHILUS* NCFM AND C-28

Test	Strain NCFM	Strain C-28	Bergey's[a]
Gram Stain	+	+	+
Cellular Morphology	rods	rods	rods
Catalase	-	-	-
Growth at 15°C	-	-	-
NH_3 from Arginine	-	-	-
Hydrolysis of Esculin	+	+	+
Acid from:			
Amygdalin	+	+	+
Arabinose	-	-	-
Cellobiose	+	+	+
Galactose	+	+	+
Glucose	+	+	+
Inositol	-	-	-
Lactose	+	+	+
Maltose	+	+	+
Mannitol	-	-	-
Mannose	+	+	+
Melezitose	-	-	-
Melebiose	-	+	±
Raffinose	+	+	±
Rhamnose	-	-	-
Salicin	+	+	+
Sorbitol	-	-	-
Sucrose	+	+	+
Trehalose	+	-	+
Xylose	-	-	-

[a]Characteristics of *L. acidophilus* as indicated in *Bergey's Manual of Determinative Bacteriology* 8th Edition (Buchanan, 1974).

TABLE II

STABILITY OF LACTOBACILLUS ACIDOPHILUS C-28
IN PASTEURIZED WHOLE MILK STORED AT 5°C

Storage Time (Days)	Colony Forming Units/ml	
	LBS agar[a]	LBSO agar[b]
1	2.1×10^7	1.6×10^7
3	1.7×10^7	1.6×10^7
5	2.1×10^7	1.5×10^7
7	1.9×10^7	1.4×10^7

[a]Number of facultative lactobacilli.

[b]Number of bile-resistant facultative lactobacilli.

TABLE III

NUMBERS OF LACTOBACILLI IN MILK CONTAINING *LACTOBACILLUS ACIDOPHILUS*

Week	Lactobacilli/ml[a]	
	L. acidophilus NCFM	*L. acidophilus* C-28
1[b]	8.8×10^6	1.4×10^7
2	1.0×10^7	1.5×10^7
3	1.8×10^6	2.0×10^7
4	2.0×10^6	8.7×10^6
5	2.7×10^6	9.0×10^6
6	2.8×10^6	9.0×10^6
7	7.6×10^6	3.5×10^6
8	6.0×10^6	2.8×10^6
9	5.6×10^6	4.4×10^6
10	9.2×10^6	1.4×10^7

[a]Determined by plating on LBS agar.

[b]Fresh batches prepared each week; counts were determined on day each batch was prepared.

(week 3) and low as 2.8×10^6/ml (week 8). The numbers of lactobacilli in the milk prepared with L. acidophilus NCFM varied from 1.0×10^7/ml (week 2) to 1.8×10^6/ml (week 3).

Analysis of Colostrum

Of five lots of colostrum, the first two contained excessive numbers of coliform bacteria (Table IV). These two lots were excluded from the feeding experiments.

Feeding Trial I

The results from the microbiological examination of fecal samples taken on days 1, 7, and 14 are presented in tabular form (Table V and VI). The counts of the facultative lactobacilli and coliform bacteria are presented as $\log_{10}$ of colony forming units per gram of dry weight of filterable fecal solids. The counts for individual calves and the average counts for each group of calves are presented.

Facultative Lactobacilli

There were wide variations in the numbers of facultative lactobacilli among the fecal samples from individual calves in each group (Table V). This was noted particularly for the 1 day old calves. The variation was less among samples on day 7 and 14 in the groups being fed L. acidophilus.

The differences in counts noted between days 1 and 7, day 1 and 14, and between days 7 and 14 are shown in Table VI. The average numbers of lactobacilli in the feces of the calves increased during the 14-day feeding period for all three groups. The increases in the

TABLE IV

ANALYSIS OF COLOSTRUM FOR COLIFORM CONTENT

Lot	Coliforms/ml[a]
1	2.7×10^6
2	1.1×10^5
3	3.8×10^3
4	3.2×10^3
5	3.5×10^4

TABLE V

INFLUENCE OF FEEDING CELLS OF *LACTOBACILLUS ACIDOPHILUS* ON NUMBERS OF FACULTATIVE LACTOBACILLI IN THE FECES OF CALVES (TRIAL I)

Group	Calf	Colony Forming Units/g[a]		
		Day 1	Day 7	Day 14
Control	01	5.40	5.96	7.04
	02	7.81	9.28	6.15
	11	7.79	8.46	9.60
	14	9.63	8.85	10.26
	18	6.87	8.66	7.93
	Average	7.50	8.24	8.20
	Standard Deviation	1.54	1.31	1.71
L. acidophilus NCFM	05	<3.26	6.96	7.34
	12	8.11	8.08	9.84
	16	7.99	7.23	9.00
	19	7.53	8.56	8.83
	20	8.04	8.56	7.86
	Average	6.99	7.88	8.57
	Standard Deviation	2.10	0.75	0.98
L. acidophilus C-28	04	6.97	9.23	9.33
	08	6.93	10.04	10.00
	10	8.04	8.20	8.60
	13	7.90	7.91	9.43
	17	4.38	8.76	8.48
	Average	6.84	8.83	9.17
	Standard Deviation	1.47	0.85	0.63

[a]Data presented as $\log_{10}$ of number of lactobacilli per gram of dry weight of filterable fecal solids.

TABLE VI

INFLUENCE OF FEEDING CELLS OF *LACTOBACILLUS ACIDOPHILUS* ON CHANGES IN THE NUMBERS OF FACULTATIVE LACTOBACILLI IN THE FECES OF CALVES (FEEDING TRIAL I)

Group	Calf	Change in Colony Forming Units/g[a]		
		Days 1 to 7	Days 1 to 14	Days 1 to 14
Control	01	+0.56	+1.64	+1.08
	02	+1.47	-1.66	-3.13
	11	+0.67	+1.81	+1.14
	14	-0.78	+0.63	+1.41
	18	+1.79	+1.06	-0.73
	Average	+0.74	+0.70	-0.05
L. acidophilus NCFM	05	+3.70	+4.08	+0.38
	12	-0.03	+1.73	+1.76
	16	-0.76	+1.01	+1.77
	19	+1.03	+1.30	+0.27
	20	+0.52	-0.18	-0.70
	Average	+0.89	+1.59	+0.70
L. acidophilus C-28	04	+2.26	+2.36	+0.10
	08	+3.11	+3.07	+0.04
	10	+0.16	+0.54	+0.40
	13	+0.01	+1.53	+1.52
	17	+4.38	+4.10	-0.28
	Average	+1.98	+2.32	+0.36

control group probably represent the normal development of the intestinal flora in neonatal calves. The average numbers in the groups which were fed L. acidophilus indicated greater increases from days 1 to 7 and from days 1 to 14 than in the control group. The group receiving milk containing the strain C-28 (isolated from the intestinal contents of a calf) exhibited the greatest increases during these periods.

An analysis of variance of the numbers of lactobacilli detected in the fecal samples on days 1, 7, and 14 (Table V) is shown in Table VII. The analysis showed no significant effect due to feeding the calves milk containing L. acidophilus ($P = 0.781$). There was also no interaction between days and treatment ($P = 0.568$). However, there was a day effect ($P = 0.001$) indicating that the increases observed as the calf aged were significant.

Since the treatments could not have affected the day 1 counts, the data was also analyzed as changes (increases or decreases) in numbers of lactobacilli in the feces samples taken on day 7 and 14 during the feeding trial (Table VI). The analysis of variance of this data is in Table VIII. The data was analyzed as changes from day 1 to day 7; day 1 to day 14; and day 7 to day 14. Even though the group averages (Table VI) showed larger increases in numbers of lactobacilli in feces of calves being fed L. acidophilus (day 1 to 7 and day 1 to 14), these changes were not significantly different from increases for the control group.

Coliform Bacteria

The numbers of coliforms varied between the individual calves (Table IX). The greatest variation among individual animals appeared

TABLE VII

ANALYSIS OF VARIANCE OF THE INFLUENCE OF FEEDING *LACTOBACILLUS ACIDOPHILUS* ON NUMBERS OF FACULTATIVE LACTOBACILLI IN THE FECES OF CALVES IN FEEDING TRIAL I

Test	Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Probability > F
	Corrected total	44	90.501	2.507		
Numerator	Treatment	2	1.683	0.841	0.255	0.781
Denominator	Calf * Treatment	12	39.588	3.299		
Numerator	Day	2	19.613	9.807	9.262	0.001
Denominator	Calf * Day (Treatment)	24	25.410	1.059		
Numerator	Treatment * Day	4	4.206	1.502	0.993	0.568
Denominator	Calf * Day (Treatment)	24	25.410	1.059		

TABLE VIII

ANALYSIS OF VARIANCE OF THE INFLUENCE OF FEEDING LACTOBACILLUS ACIDOPHILUS ON CHANGES IN THE NUMBERS OF FACULTATIVE LACTOBACILLI IN THE FECES OF CALVES IN FEEDING TRIAL I

Time Period	Test	Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Probability > F
		Total	14	34.059	2.465		
Day 1 to Day 7	Numerator	Treatment	2	4.596	2.298	0.922	0.573
	Denominator	Calf * Treatment	12	29.913	2.493		
		Total	14	31.693	2.264		
Day 1 to Day 14	Numerator	Treatment	2	6.646	3.323	1.592	0.243
	Denominator	Calf * Treatment	12	25.047	2.087		
		Total	14	22.649	1.618		
Day 7 to Day 14	Numerator	Treatment	2	1.377	0.689	0.388	0.691
	Denominator	Calf * Treatment	12	21.271	1.773		

TABLE IX

INFLUENCE OF FEEDING CELLS OF *LACTOBACILLUS ACIDOPHILUS* ON NUMBERS OF COLIFORM BACTERIA IN THE FECES OF CALVES (TRIAL I)

Group	Calf	Colony Forming Units/g[a]		
		Day 1	Day 7	Day 14
Control	01	8.11	9.91	9.93
	02	9.90	9.64	9.08
	11	9.65	9.51	8.58
	14	10.49	9.92	9.63
	18	9.46	8.20	8.85
	Average	9.52	9.44	9.21
	Standard Deviation	0.88	0.71	0.56
L. acidophilus NCFM	05	9.34	8.67	7.97
	12		9.48	8.26
	16	8.95	9.58	9.18
	19	10.38	9.88	8.98
	20	9.99	9.68	8.90
	Average	9.67	9.48	8.67
	Standard Deviation	0.56	0.47	0.52
L. acidophilus C-28	04	10.46	9.32	9.46
	08	10.15	9.23	8.77
	10	9.20	9.63	8.95
	13	10.63	9.57	7.90
	17	7.38	9.26	9.40
	Average	9.56	9.40	8.90
	Standard Deviation	1.33	0.18	0.63

[a]Data presented as $\log_{10}$ of number of coliform bacteria per gram of dry weight of filterable fecal solids.

on day 1 in the group which received L. acidophilus C-28. There were decreases in the averages of the numbers of coliforms in all groups during the 14 day feeding period (Table X). The averages exhibited the greatest decrease in the two groups receiving milk that contained cells of L. acidophilus. The group fed strain NCFM from a human had the greatest reduction in the numbers of coliform bacteria in the feces.

The differences in counts noted between days 1 and 7, day 1 and 14, and between days 7 and 14 are shown in Table X. The average numbers of coliforms in the feces decreased during the 14-day feeding period for all three groups of calves. The reductions in the number of coliforms in the control group probably represent the normal changes that occur in the intestinal flora of a developing calf. The average numbers in the calves for L. acidophilus indicated greater reductions from days 1 to 7 and from day 1 to 14 than in the control group. The group receiving the milk containing the strain NCFM exhibited the greatest decreases in the numbers of coliforms during these periods.

An analyses of variance of the numbers of coliforms detected in the feces of the calves on days 1, 7, and 14 (Table IX) is shown in Table XI. The analysis showed no significant effect due to feeding the calves milk containing L. acidophilus ($P = 0.856$). There was also no interaction between treatment and days ($P = 0.862$). However, there was a significant day effect ($P = 0.049$) indicating that the decreases observed as the calf aged were significant.

Since the treatments could not have affected the counts on day 1, the results were analyzed for significant increases or decreases resulting from feeding milk containing L. acidophilus. The analysis of variance for this data is in Table XII. The data was

TABLE X

INFLUENCE OF FEEDING CELLS OF *LACTOBACILLUS ACIDOPHILUS* ON CHANGES IN THE NUMBERS OF COLIFORM BACTERIA IN THE FECES OF CALVES (TRIAL I)

Group	Calf	Change in Colony Forming Units/g[a]		
		Day 1 to Day 7	Day 1 to Day 14	Day 7 to Day 14
Control	01	+1.80	+1.82	+0.02
	02	-0.26	-0.82	-0.56
	11	-0.14	-1.07	-0.93
	14	-0.57	-0.84	-0.29
	18	-1.26	-0.61	+0.65
	Average	-0.09	-0.30	-0.22
L. acidophilus NCFM	05	-0.67	-1.37	-0.70
	12			-1.22
	16	+0.63	+0.23	-0.40
	19	-0.50	-1.40	-0.90
	20	-0.31	-1.09	-0.78
	Average	-0.21	-0.90	-0.80
L. acidophilus C-28	04	-1.14	-1.00	+0.14
	08	-0.92	-1.38	-0.46
	10	+0.40	-0.25	-0.68
	13	-1.06	-2.73	-1.67
	17	+1.88	+2.02	-0.14
	Average	-0.17	-0.67	-0.56

[a]Reduction in coliform bacteria indicated as (-). Increase in coliform bacteria indicated as (+).

TABLE XI

ANALYSIS OF VARIANCE OF THE INFLUENCE OF FEEDING *LACTOBACILLUS ACIDOPHILUS* ON NUMBERS OF COLIFORM BACTERIA IN THE FECES OF CALVES IN FEEDING TRIAL I

Test	Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Probability > F
	Corrected total	44	22.650	0.515		
Numerator	Treatment	2	0.159	0.080	0.016	0.856
Denominator	Calf * Treatment	12	6.039	0.503		
Numerator	Day	2	3.489	1.745	3.402	0.049
Denominator	Calf * Day * Treatment	24	12.308	0.513		
Numerator	Treatment * Day	4	0.655	0.165	0.319	0.862
Denominator	Calf * Day * Treatment	24	12.308	0.513		

TABLE XII

ANALYSIS OF VARIANCE OF THE INFLUENCE OF FEEDING LACTOBACILLUS ACIDOPHILUS ON CHANGES IN NUMBERS OF COLIFORM BACTERIA IN THE FECES OF CALVES IN FEEDING TRIAL I

Testing Period	Test	Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Probability > F
		Corrected Total	14	13.061	0.933		
Day 1 to Day 7	Numerator	Treatment	2	0.015	0.008	0.007	0.994
	Denominator	Calf * Treatment	12	13.045	1.087		
Day 1 to Day 14	Numerator	Treatment	2	1.060	0.530	0.320	0.736
	Denominator	Calf * Treatment	12	19.871	1.656		
Day 7 to Day 14	Numerator	Treatment	2	0.889	0.444	1.331	0.301
	Denominator	Calf * Treatment	12	4.006	0.334		

evaluated as changes from day 1 to day 7; day 1 to day 14; and day 7 to day 14. The groups averages (Table X) showed larger decreases in numbers of coliforms in calves being fed milk containing L. acidophilus (day 1 to 7 and day 1 to day 14). However, these decreases were not significantly different from the decreases in coliform in the control group.

Feeding Trial II

The results from the microbiological examination of the individual fecal and intestinal samples are presented in tabular form (Tables XIII and XIV). The bacterial counts are expressed as $\log_{10}$ of the count per gram dry weight of filterable fecal or intestinal solids.

Facultative Lactobacilli

As in trial 1, there were wide variations in numbers of lactobacilli among the calves in each group (Table XIII). This was true of both the fecal samples and the intestinal segments. The amounts of variation in all groups on day 1 were similar. The variation in the numbers of lactobacilli in the feces from the group C-28 were considerably less on day 7 than for the other groups. The average number of lactobacilli in feces on day 7 and in the small intestines of the calves in the group which was fed L. acidophilus C-28 were higher than in either of the other groups. Little difference was observed in the average numbers in the large intestines.

The numbers of lactobacilli in the large intestines of all calves except for number 29 were greater than those detected in the small intestine. This difference was greater for the control group than the

TABLE XIII

INFLUENCE OF FEEDING CELLS OF *LACTOBACILLUS ACIDOPHILUS* ON NUMBERS OF FACULTATIVE LACTOBACILLI IN THE FECES AND INTESTINES OF CALVES (TRIAL II)

Group	Calf	Colony Forming Units/g[a]			
		Feces		Intestines	
		Day 1	Day 7	Small	Large
Control	25	7.99	8.08	6.80	7.83
	27	4.71	9.72	6.11	8.80
	30	7.78	9.86	7.23	9.15
	36	8.40	7.23	6.07	8.46
	Average	7.22	8.72	6.55	8.56
	Standard Deviation	1.69	1.28	0.56	0.56
L. acidophilus NCFM	22	8.34	8.87	8.18	9.96
	28	6.04	7.18	6.36	6.63
	33	7.70	9.65	6.43	8.75
	39	7.11	7.96	6.80	8.43
	Average	7.30	8.42	6.94	8.44
	Standard Deviation	1.52	1.07	0.85	1.38
L. acidophilus C-28	24	5.58	8.61	6.62	9.23
	29	6.36	9.26	8.00	7.32
	32	7.76	9.56	7.67	8.98
	38	8.79	8.81	7.56	8.51
	Average	7.12	9.06	7.46	8.51
	Standard Deviation	1.43	0.43	0.59	0.85

[a]Data presented as $\log_{10}$ of number of lactobacilli per gram of dry weight of filterable fecal or intestinal solids.

group which were fed cells of L. acidophilus. However, the small intestines of the calves fed L. acidophilus contained higher numbers of lactobacilli than the control group.

The differences (increases or decreases) in counts in the feces between days 1 and 7 are presented in Table XIV. The numbers of lactobacilli detected in the feces of all groups increased during the 7 day feeding period. The increase was most evident in the group which received milk containing L. acidophilus C-28. The numbers of lactobacilli in the control group increased greater than in the group receiving L. acidophilus NCFM. However, the small intestines of the calves fed L. acidophilus contained higher number of lactobacilli than the control group.

An analysis of variance of the results obtained from enumeration of the bactobacilli in fecal samples on day 1 and 7 appears in Table XV. As in the first trial, there was a significant ($P = 0.016$) increase in numbers of lacobacilli from day 1 to day 7 for all groups. However, the numbers in the two groups that were fed L. acidophilus were not significantly different ($P = 0.922$) from the number in the control group. Analysis of variance of the changes in numbers of lactobacilli from day 1 to day 7 is shown in Table XVI. The changes in numbers of lactobacilli among groups were not significantly ($P = 0.815$) different. Feeding L. acidophilus to the calves did not significantly ($P = 0.652$) affect the numbers of lactobacilli in the intestines (Table XVII). There was, however, significantly ($P = 0.001$) more lactobacilli in the large intestines than in the small intestines. No significant ($P = 0.530$) interaction between treatment and location (i.e. small or large intestine) was evident.

TABLE XIV

INFLUENCE OF FEEDING CELLS OF LACTOBACILLUS ACIDOPHILUS ON CHANGES IN THE NUMBERS OF FACULTATIVE LACTOBACILLI IN THE FECES OF CALVES (FEEDING TRIAL II)

		Changes in Colony Forming Units/g[a]
		Feces
Group	Calf	Day 1 to Day 7
Control	25	+0.09
	27	+5.01
	30	+2.08
	36	-1.17
	Average	+1.50
L. acidophilus NCFM	22	+0.53
	28	+1.18
	33	+1.95
	39	+0.85
	Average	+1.13
L. acidophilus C-28	24	+3.03
	29	+2.90
	32	+1.80
	38	+0.02
	Average	+1.94

[a]Reduction in lactobacilli indicated as (-). Increase in lactobacilli indicated as (+).

TABLE XV

ANALYSIS OF VARIANCE OF THE INFLUENCE OF FEEDING *LACTOBACILLUS ACIDOPHILUS* ON NUMBERS OF FACULTATIVE LACTOBACILLI IN THE FECES OF CALVES IN FEEDING TRIAL II

Test	Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Probability > F
	Corrected Total	23	41.302	1.796		
Numerator	Treatment	2	0.221	0.110	0.081	0.922
Denominator	Calf * Treatment	9	12.0221	1.357		
Numerator	Day	1	13.847	13.847	8.684	0.016
Denominator	Calf * Day	9	14.350	1.594		
Numerator	Treatment * Day	2	0.673	0.337	0.211	0.815
Denominator	Calf * Day * Treatment	9	14.350	1.594		

TABLE XVI

ANALYSIS OF VARIANCE OF THE INFLUENCE OF FEEDING LACTOBACILLUS ACIDOPHILUS ON CHANGES IN NUMBERS OF FACULTATIVE LACTOBACILLI IN THE FECES OF CALVES IN FEEDING TRIAL II

Time Period	Test	Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Probability > F
		Corrected Total	11	30.046	2.731		
Day 1 to Day 7	Numerator	Treatment	2	1.346	0.673	0.211	0.815
	Denominator	Calf * Treatment	9	28.699	3.189		

TABLE XVII

ANALYSIS OF VARIANCE OF THE INFLUENCE OF FEEDING LACTOBACILLUS ACIDOPHILUS ON NUMBERS OF FACULTATIVE LACTOBACILLI IN THE INTESTINES OF CALVES IN FEEDING TRIAL II

Test	Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Probability > F
	Corrected Total	23	23.683	1.247		
Numerator	Treatment	2	6.837	0.418	0.455	0.652
Denominator	Calf * Treatment	9	8.280	0.920		
Numerator	Location	1	14.030	14.030	27.018	0.001
Denominator	Calf * Location * Treatment	9	4.674	0.519		
Numerator	Treatment * Location	2	0.863	0.431	0.831	0.530
Denominator	Calf * Location * Treatment	9	4.674	0.519		

Coliform Bacteria

The numbers of coliform bacteria in the feces varied widely between individual calves in each group (Table XVIII). The 7 day old calves which had been fed strain NCFM had the smallest amount of variation in numbers of coliform bacteria in their feces. In general the numbers of coliforms were lower on day 7.

Greater numbers of coliforms were detected in the large intestine than in the small intestine of all calves except for calf number 38. The average numbers of coliforms were lower in the calves receiving L. acidophilus than in the control calves. An exception to this was the large intestines of the calves receiving strain NCFM.

The changes in numbers of lactobacilli in the feces of the calves from day 1 to day 7 are shown in Table XIX. The average decrease from day 1 to day 7 was greatest for the calves receiving milk containing L. acidophilus C-28. The milk containing strain NCFM appeared not to effect the numbers of coliform bacteria in the feces.

An analysis of variance of the numbers of coliforms detected in the feces of the calves on days 1 and 7 (Table XVIII) is shown in Table XX. The analysis showed no significant effect due to feeding milk containing L. acidophilus to the calves ($P = 0.676$). There was also no interaction between treatment and days ($P = 0.173$). However, as in trial 1, there was a significant day effect ($P = 0.023$).

The results were analyzed for significant increases or decreases in numbers of coliform bacteria resulting from feeding milk containing L. acidophilus (Table XIX). The analysis of variance for this data is in Table XXI. The data was evaluated as changes from day 1 to day 7. Even though the group averages (Table XIII) showed larger decreases in

TABLE XVIII

INFLUENCE OF FEEDING CELLS OF *LACTOBACILLUS ACIDOPHILUS* ON NUMBERS OF COLIFORM BACTERIA IN THE FECES AND INTESTINES OF CALVES (TRIAL II)

Group	Calf	Colony Forming Units/g[a]			
		Feces		Intestines	
		Day 1	Day 7	Small	Large
Control	25	9.84	9.40	5.34	7.83
	27	8.99	9.34	5.53	8.80
	30	10.11	10.08	7.15	8.66
	36	10.23	9.61	6.15	9.83
	Average	9.79	9.61	6.04	8.78
	Standard Deviation	0.68	0.40	0.81	1.46
L. acidophilus NCFM	22	9.81	9.46	5.26	8.20
	28	8.92	9.51	4.83	9.46
	33	10.04	9.52	4.95	8.63
	39	9.73	9.40	6.81	9.32
	Average	9.63	9.47	5.46	8.90
	Standard Deviation	0.49	0.05	0.84	0.59
L. acidophilus C-28	24	9.28	8.67	4.75	9.48
	29	9.92	9.75	5.88	6.30
	32	10.18	8.99	5.81	7.00
	38	10.08	9.00	5.88	5.38
	Average	9.87	9.10	5.58	7.04
	Standard Deviation	0.40	0.46	0.55	1.74

[a]Data presented as a $\log_{10}$ of number of coliform bacteria per gram of dry weight of filterable fecal or intestinal solids.

TABLE XIX

CHANGES IN THE NUMBER OF COLIFORM BACTERIA IN THE FECES OF THE CALVES OF FEEDING TRIAL II

Group	Calf	Changes in Colony Forming Units/g[a] Feces Day 1 to Day 7
Control	25	-0.44
	27	+0.35
	30	-0.03
	36	-0.62
	Average	-0.18
L. acidophilus NCFM	22	-0.35
	28	+0.59
	33	-0.52
	39	-0.33
	Average	-0.15
L. acidophilus C-28	24	-0.61
	29	-0.17
	32	-1.19
	38	-1.08
	Average	-0.76

[a]Reduction in coliform bacteria indicated as (-). Increase in coliform bacteria indicated as (+).

TABLE XX

ANALYSIS OF VARIANCE OF THE INFLUENCE OF FEEDING LACTOBACILLUS ACIDOPHILUS ON NUMBERS OF COLIFORM BACTERIA IN THE FECES OF CALVES IN FEEDING TRIAL II

Test	Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Probability > F
	Corrected Total	23	4.595	0.200		
Numerator	Treatment	2	0.197	0.098	0.416	0.676
Denominator	Calf * Treatment	9	2.130	0.237		
Numerator	Day	1	0.807	0.807	7.336	0.023
Denominator	Calf * Day	9	0.990	0.110		
Numerator	Treatment * Day	2	0.417	0.236	2.14	0.173
Denominator	Calf * Day * Treatment	9	0.990	0.110		

TABLE XXI

ANALYSIS OF VARIANCE OF THE INFLUENCE OF FEEDING LACTOBACILLUS ACIDOPHILUS ON CHANGES IN NUMBERS OF COLIFORM BACTERIA IN THE FECES OF CALVES IN FEEDING TRIAL II

Time Period	Test	Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Probability > F
		Corrected Total	11	2.921	0.266		
Day 1 to Day 7	Numerator	Treatment	2	6.942	0.471	2.142	0.173
	Denominator	Calf * Treatment	9	1.979	0.220		

numbers of coliforms in feces of calves being fed L. acidophilus, these changes were not significantly different from the decreases for the control group.

Feeding L. acidophilus to the calves did not significantly (P = 0.081) affect the numbers of coliforms in the intestines (Table XXII). There was, however, as in trial 1, a highly significant (P = 0.001) difference in the numbers of coliforms in the small and large intestines. There was no significant (P = 0.232) interaction between treatment and location (i.e. small or large intestine).

Predominating Facultative Lactobacilli in Feces

The predominating types of facultative lactobacilli in the feces of the 7 day old calves in both feeding trials were isolated and identified. The identities of the isolates from the individual fecal samples are presented in Tables XXV and XXVI (Appendix). The predominating types of facultative lactobacilli in the fecal samples were: L. acidophilus; L. fermentum; L. cellobiosus; and L. salivarus.

The numbers of L. acidophilus isolated on day 7 from all fecal samples of individual calves in both feeding trials are shown in Table XXIII. The milk containing L. acidophilus C-28 appeared to greatly enhance the incidence of the organism in the feces (an average of 4.2 L. acidophilus isolated/sample). The number of L. acidophilus (average of 1.9/sample) recovered from the group fed strain NCFM was much less and was about the same as the number (average of 1.8/sample) isolated from the control group.

Analysis of variance of the data (Table XXIV) indicated significant variation ($P < 0.025$) among groups with respect to numbers of

TABLE XXII

ANALYSIS OF VARIANCE OF THE INFLUENCE OF FEEDING *LACTOBACILLUS ACIDOPHILUS* ON NUMBERS OF COLIFORM BACTERIA IN THE INTESTINES OF CALVES IN FEEDING TRIAL II

Test	Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Probability > F
	Corrected Total	23	66.088	2.873		
Numerator	Treatment	2	5,404	2.702	3.350	0.081
Denominator	Calf * Treatment	9	7.258	0.806		
Numerator	Location	1	33.888	33.888	33.304	0.001
Denominator	Calf * Location * Treatment	9	10.509	1.168		
	Location	2	4.031	2.015	1.726	0.232
	Calf * Location * Treatment	9	10.509	1.168		

TABLE XXIII

INCIDENCE OF *LACTOBACILLUS ACIDOPHILUS* AMONG LACTOBACILLI IN FECES OF 7 DAY OLD CALVES

Group	Calf	Number of *L. acidophilus*[a]
Control	01	0
	02	0
	11	0
	14	3
	18	5
	25	0
	27	4
	30	2
	36	2
	Average	1.8
L. acidophilus NCFM	05	0
	12	5
	16	4
	19	0
	20	0
	22	0
	28	4
	33	2
	39	2
	Average	1.9
L. acidophilus C-28	04	5
	08	5
	10	2
	13	5
	17	5
	24	3
	29	5
	32	3
	38	5
	Average	4.2

[a]Number of *L. acidophilus* isolated from a total of 5 colonies from LBS agar for each sample.

TABLE XXIV

ANALYSIS OF VARIANCE OF THE INFLUENCE OF FEEDING LACTOBACILLUS ACIDOPHILUS ON OCCURRENCE OF LACTOBACILLUS ACIDOPHILUS IN FECES OF 7 DAY OLD CALVES

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Value
Total	26	108.296		
Treatment	2	34.295	17.148	5.56[a]
Calf * Treatment	24	74.001	3.083	

[a]Significant at $P < 0.025$ (F value of 4.32 required for significance at 0.025 level).

L. acidophilus. Thus feeding milk containing L. acidophilus C-28 to calves resulted in a significant increase in the occurrence of L. acidophilus in their feces. Feeding milk containing L. acidophilus NCFM did not have a similar effect.

CHAPTER V

DISCUSSION

Many investigators have studied the concept of establishing L. acidophilus in the intestinal tract to aid in the maintenance of a favorable microflora. Gastrointestinal disorders may be caused by an upset in the normal microbial balance (Zubrzycki and Spaulding, 1962). The consumption of the organism was initially proposed as a therapeutic treatment for various gastrointestinal disturbances and as an aid in helping the intestinal flora return to a normal balance after antibiotic therapy (Kopeloff, 1926; Beck and Necheles, 1961; Macbeth et al., 1965). The primary method of administering the organism was the consumption of milk fermented by L. acidophilus. However, the product possessed a highly objectionable flavor and did not obtain public acceptance. Myers (1931) showed that consumption of an unfermented milk containing cells of L. acidophilus which had been grown in broth caused increases in the numbers of lactobacilli in the intestinal tract of humans. The unfermented milk possessed the same flavor as that of fresh milk. The technology of mass producing concentrated cultures of the lactobacilli was not available at that time so the product was not commercially produced. In the early 1970's such a commercial product was produced. Gilliland et al. (1978) showed consumption of such a product resulted in increased numbers of lactobacilli in the intestinal contents of humans. The milk containing L. acidiphilus

used in the present study was prepared in a similar manner to that reported by Gilliland et al. (1978).

In this study, concentrated cell suspensions of L. acidophilus NCFM of a human origin and L. acidophilus C-28 of a calf origin were used to prepare the milk. This was done since several investigators have suggested that L. acidophilus exhibits a host-specific relationship among strains of the organism (Mitusoka, 1968, Morishita, 1971; Gilliland et al., 1975). The identity of both cultures was confirmed as being L. acidophilus. This is essential if such cultures are to be properly evaluated for their function in the intestinal tract since not all species of lactobacilli will survive in the intestinal tract (Gilliland, 1979).

The numbers of facultative lactobacilli in the feces of the calves in all groups increased significantly during the feeding experiments. This was accompanied by reductions in the numbers of coliform bacteria. The feeding of milk containing L. acidophilus did not have a statistically significant effect on the numbers of lactobacilli and coliforms in the feces of the calves. Even though the differences were not statistically significant there was a trend toward higher numbers of lactobacilli and lower numbers of coliforms in the calves which received L. acidophilus. Furthermore, the intestinal flora of the calves was developing rapidly as evidenced by the counts for lactobacilli and coliform. Since the flora in the control animals was changing so much it may have been difficult to observe a great effect by feeding L. acidophilus. This is especially true since the animals were healthy and the lactobacilli were apparently increasing rapidly in the control animals. The numbers of lactobacilli did seem to increase more rapidly in the animals receiving

L. acidophilus C-28. Several studies have shown more drastic declines in the numbers of coliforms after feeding L. acidophilus to humans (Tomic - Karovic and Fanjek, 1962; Read et al., 1966). However, in these studies the test subjects were not healthy in that they were suffering from diarrhea. The cells were fed as a therapeutic agent for the treatment of their illness. Chopra et al. (1963) noted that the coliforms outnumbered the lactobacilli in the intestines of scouring pigs.

The effect of feeding milk containing strain C-28 was even more apparent when the identities of the predominating lactobacilli in the feces of the animals were compared. The calves that received L. acidophilus C-28 exhibited significantly more L. acidophilus in their feces than did the calves that received strain NCFM and the control group. This indicates that L. acidophilus of calf origin more effectively established in the intestinal tract of the calf than did the one of human origin. The lack of establishment of L. acidophilus NCFM in the calves may have been due to its not being as compatible as strain C-28 with the intestinal tract of the calf. This concept is supported in a previous study in which a strain of L. acidophilus isolated from a human failed to be established in the intestinal tract of a germ-free chicken (Morishita et al., 1971). However, he showed that a strain of L. acidophilus isolated from a conventional chicken was easily established in the intestinal tract of the germ-free chicken.

The reductions in numbers of coliform bacteria in the intestinal flora accompanied by a concurrent increase in numbers of lactobacilli is apparently a normal happening in the development of the intestinal flora of calves. While the reductions in numbers of coliform bacteria

were not as great as the increases in numbers of lactobacilli it does suggest that the lactobacilli in the developing intestinal flora exerts a controlling effect on the coliform bacteria. Several investigators have observed an increase in the numbers of lactobacilli accompanying a decrease in the levels of coliforms after oral administration of L. acidophilus (Sandine et al., 1972; Gilliland et al., 1978; Gilliland, (1979).

The number of lactobacilli and levels of coliforms in the control group were comparable to the levels observed by Smith and Crabb (1961). The calves that were fed L. acidophilus C-28 in general possessed fewer coliforms in the intestinal tract than did those in the other groups. This may have been due to it being more effective than strain NCFM in establishing in the intestines of the calf.

Various species of lactobacilli other than L. acidophilus were observed to be part of the lactobacillus flora in the feces of the calves. Other species included: L. fermentum; L. salivarus; and L. cellobiosus. All of these species as well as L. acidophilus are resistant to bile (Buchanan, 1974). Gilliland (1979) indicated that lactobacilli should be resistant to bile salts if they are to survive and establish in the intestinal tract. It is possible that species of intestinal lactobacilli, in addition to the L. acidophilus, may be important in maintaining a desirable balance of the microbial flora. The feeding of. L. acidophilus to the calves did not have a significant effect on the numbers of intestinal organisms examined. Administration of milk containing higher numbers of L. acidophilus may have caused larger differences in the numbers of lactobacilli and coliforms in the intestines of the calves. This is especially true for the calves that

were fed milk containing strain NCFM since these calves actually received fewer cells of L. acidophilus than did the calves fed milk containing strain C-28.

An important consideration in future studies is to feed higher numbers of the organism. Gilliland et al. (1978) showed that the numbers of L. acidophilus in milk fed to healthy human males affected the numbers of facultative lactobacilli appearing in their feces. The strains of L. acidophilus fed to the calves should also be prepared in milk in such a manner to insure equal numbers of the organism. This would provide more definitive evidence related to host specificity of L. acidophilus.

One of the main reasons for using L. acidophilus as a dietary adjunct is to control intestinal pathogens such as species of Salmonella and enteropathogenic Escherichia coli. To obtain needed information on the possible role of L. acidophilus in controlling these pathogens in the intestines of animals and humans, studies are needed in which the test subjects being fed L. acidophilus are challenged with the intestinal pathogens. Such challenge experiments should be included in future studies.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Two strains of L. acidophilus were fed to neonatal dairy calves in two feeding trials. Fecal samples were obtained on days 1, 7, and 14 from the calves of the feeding trial. In addition to obtaining fecal samples on days 1 and 7, intestinal segments were surgically removed from the calves of the second feeding trial on day 10. For each sample, selective media were used to enumerate the populations of coliforms and facultative lactobacilli. The predominant types of lactobacilli in each fecal sample collected on day 7 were identified.

There were wide variations among calves with respect to the numbers of organisms in the intestinal microflora. There was a significant ($P < 0.05$) increase in the numbers of lactobacilli in all calves following day 1. The calves fed the milk containing L. acidophilus C-28 had a higher number of facultative lactobacilli in their intestines and feces than calves in the control group or than those fed L. acidophilus NCFM. However, these differences were not significant. The occurrence of L. acidophilus was significantly higher in feces from calves that had consumed milk containing strain C-28 than in those of the other two groups. There was a significant ($P < 0.05$) reduction in the numbers of coliforms in all calves following day 1. The numbers tended to be lower in calves that received L. acidophilus; however, the differences among treatments were not statistically significant.

The intestinal flora of the calves was developing rapidly as evidenced by the counts for lactobacilli and coliforms in the control group. Since the intestinal tract of the control animals was changing rapidly it may have been difficult to obtain a significant effect by feeding L. acidophilus. Furthermore, since the animals were healthy and they possibly had a proper balance of intestinal flora. The organism was not as effective in changing the intestinal flora than it might have if it was used as a therapeutic treatment of scours. However, calves that were fed a strain of L. acidophilus of calf origin exhibited significantly ($P < 0.05$) more L. acidophilus in their feces than did the calves that received a L. acidophilus of human origin or than did the control group. This indicates that feeding L. acidophilus can have a significant effect on the intestinal flora. Furthermore, there is an apparent host-specifity that exists among strains of the organism. This suggests that care should be taken in selecting strains of L. acidophilus for use as dietary adjuncts to insure that the selected strain will function or establish in the host animal.

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APPENDIX

TABLE XXV

IDENTITY OF PREDOMINANT LACTOBACILLI IN THE FECES OF 7 DAY OLD CALVES IN FEEDING TRIAL I

Group					
Control		*L. acidophilus* NCFM		*L. acidophilus* C-28	
Calf	Species	Calf	Species	Calf	Species
01	cellobiosus	05	salivarus	04	acidophilus
	cellobiosus		salivarus		acidophilus
	cellobiosus		salivarus		acidophilus
	cellobiosus		salivarus		acidophilus
	cellobiosus				acidophilus
02	fermentum	12	acidophilus	08	acidophilus
	fermentum		acidophilus		acidophilus
	fermentum		acidophilus		acidophilus
	fermentum		acidophilus		acidophilus
	fermentum		acidophilus		acidophilus
11	salivarus	16	acidophilus	10	salivarus
	salivarus		acidophilus		salivarus
	salivarus		acidophilus		salivarus
	salivarus		acidophilus		acidophilus
	salivarus		salivarus		acidophilus
14	acidophilus	19	fermentum	13	acidophilus
	acidophilus		fermentum		acidophilus
	acidophilus		fermentum		acidophilus
			fermentum		acidophilus
			fermentum		acidophilus
18	acidophilus	20	fermentum	17	acidophilus
	acidophilus		fermentum		acidophilus
	acidophilus		fermentum		acdiophilus
	acidophilus		fermentum		acidophilus
	acidophilus				acidophilus

TABLE XXVI

IDENTITY OF PREDOMINANT LACTOBACILLI IN THE FECES OF 7 DAY OLD CALVES IN FEEDING TRIAL II

Group					
Control		*L. Acidophilus* NCFM		*L. acidophilus* C-28	
Calf	Species	Calf	Species	Calf	Species
25	fermentum	22	fermentum	24	acidophilus
	fermentum		fermentum		acidophilus
	fermentum		fermentum		acidophilus
	fermentum		fermentum		fermentum
27	acidophilus	28	acidophilus	29	acidophilus
	acidophilus		acidophilus		acidophilus
	acidophilus		acidophilus		acidophilus
	acidophilus		acidophilus		acidophilus
	fermentum		salivarus		acidophilus
30	acidophilus	33	acidophilus	32	acidophilus
	acidophilus		acidophilus		acidophilus
	fermentum		fermentum		acidophilus
			fermentum		fermentum
			salivarus		fermentum
36	fermentum	39	acidophilus	38	acidophilus
	fermentum		acidophilus		acidophilus
	fermentum		cellobiosus		acidophilus
	acidophilus		cellobiosus		acidophilus
	acidophilus				acidophilus

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