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Title: EFFECT OF FEEDING CONCENTRATES OF LACTOBACILLUS ORGANISMS ON INTESTINAL COLONIZATION BY ESCHERICHIA COLI IN SWINE

Abstract approved: Redacted for privacy
(Dr. W. E. Sandine)

A Lactobacillus species of human intestinal origin (strain MLC) used in swine feeding experiments was characterized using biochemical, genetic and serological techniques and found to be Lactobacillus lactis. Bottle feeding of the MLC strain in concentrate form ($> 10^9$ cfu/ml) resulted in a reduction in both fecal coliforms and the incidence of scouring. In one group of pigs which received concentrate for 54 days, the Lactobacillus to coliform ratio was 1280:1; in the control group the ratio was 2.3:1.

To increase the sample size, a herd of 125 swine was fed concentrates of Lactobacillus lactis MLC through the drinking water system using a water proportioner. After 90 days of such treatment, the coliform counts were reduced by 95%. The scouring incidence in the treated pigs was 13% as compared to 35% in the control group.

However numbers of fecal lactobacilli were not increased.

The influence of Lactobacillus MLC feeding on the bacterial flora of different parts of gastrointestinal tract was studied. In the case of scouring pigs, enteropathogenic Escherichia coli (EEC) were present in larger numbers in the tissue homogenate of different parts of the tract than in the lumen. The virulence of the EEC found present was confirmed by experimental infection in pigs. In control, non-scouring pigs only non-EEC were found in the tissue. In Lactobacillus MLC-fed pigs, E. coli both in the tissue and lumen was reduced to low numbers; also, the few E. coli observed were non-enteropathogenic. Thus by feeding Lactobacillus MLC concentrate, it was possible to reduce the E. coli to less than 10^2/gm. There were higher numbers of lactobacilli in the tissues of Lactobacillus-fed pigs than in control and scouring pigs. The lactobacilli isolated from tissue homogenate of the treated animals resembled biochemically and serologically (fluorescent antibody staining) the Lactobacillus MLC which was fed.

Histological studies were done to show direct evidence of colonization in frozen sections of intestine obtained from Lactobacillus MLC-fed pigs. Gram and toluidine blue-staining revealed large numbers of Gram-positive bacilli colonizing the surface epithelium of the villi. On the other hand, control pigs which died of scouring revealed many EEC colonizing the small intestine. Pigs in

groups receiving colostrum and lactobacilli performed very well. No symptoms of diarrhea was seen and many lactobacilli colonized throughout the small intestine. Even after the challenge with EEC serotype 09:K:NM, these two groups of pigs did not show any signs of disease and very few EEC colonized the intestines even after the challenge. Pigs not receiving colostrum but only lactobacilli did not scour before challenge with EEC 09:K:NM and many lactobacilli colonized the small intestine. However, 72 hours after challenge these latter animals revealed symptoms of diarrhea and EEC were seen colonizing the small intestine in addition to lactobacilli.

The possible role of surface antigens in colonization by lactobacilli was studied. Data revealed that Lactobacillus lactis MLC and L. salivarius did not have any antigens in common. On the other hand, Lactobacillus FHS isolated from pig intestine had three antigens in common with the MLC strain. However, in vivo tests showed that all three strains colonized the small intestine to the same degree. This indicated that surface antigens were not involved in the colonization mechanism.

The ability of Lactobacillus MLC to inhibit a variety of intestinal pathogens in broth cultures was demonstrated. Organisms inhibited included E. coli, Staphylococcus aureus, Clostridium perfringens and Bacteroides sp. The mechanism of inhibition of S. aureus and E. coli in milk and broth was examined. These

organisms did not grow in cell-free culture supernatants (whey at pH 4.0) after growth of the Lactobacillus MLC but they grew well in broth adjusted to pH 4.0 Supernatant from cultures of Lactobacillus MLC concentrate was found to contain 2-Deoxy-D-glucose in addition to glucose and galactose. Studies using 2-Deoxy-D-glucose alone and with glucose and galactose showed that the former was inhibitory to E. coli, S. aureus and Salmonella typhimurium.

Possible applications of these findings in the animal industry as a substitute to antibiotics are discussed. A greater use of Lactobacillus organisms in preventive treatment of intestinal diseases is suggested.

Effect of Feeding Concentrates of *Lactobacillus* Organisms on Intestinal Colonization by *Escherichia coli* in Swine

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INTRODUCTION

Intestinal infection caused by enteropathogenic *Escherichia coli* (EEC) is a major health problem in both humans and domestic animals. In fact, scouring in piglets caused by EEC is a major economic problem in the swine industry and is responsible for about 20% mortality among newborn pigs. In contrast to the birth of human infants, pigs are born into a highly contaminated environment; as a result, there is a great opportunity for EEC to colonize the intestinal tract during the early life of the pig. Colostrum, which is received in their immediate postnatal life, gives a temporary, passive protection against EEC infection. Later, due to the establishment of lactic acid bacteria in the intestine, a balance develops and is maintained between EEC and lactobacilli; this, in the normal animal, helps maintain a healthful condition in the intestinal tract.

Organisms such as *Salmonella* sp., *Vibrio* sp., and enterotoxin-producing strains of *E. coli* are well recognized as undesirable inhabitants of the gastrointestinal tract. Desirable members of the intestinal flora are less well defined but numerous studies dating from the early 1900's to the present indicate that lactic acid bacteria, especially those of the genus *Lactobacillus* and *Bifidobacterium* are

important. The precise nature of their beneficial effect, direct or indirect, remains to be determined, though studies with breast-fed infants recently carried out by Mata et al. (1972) leave little doubt that intestinal colonization by lactic acid bacteria is a major factor in minimizing enteric disease among newborn infants.

The lactic acid bacteria consist of rod shaped and spherical shaped organisms. They are widely distributed in nature. Because lactic acid is the principal end product of their carbohydrate fermentation, they have been exploited by man for thousands of years in food preservation. Despite the great importance to man which these bacteria assume, they have received, comparatively speaking, little research attention. Interest in the lactic acid bacteria has increased recently due to their role in maintaining a proper microbial balance in the intestinal tract. Their importance both in human and animal health and their possible ability to complement or replace certain antibiotic treatments is receiving increased research attention today.

Metchnikoff (1908) was intrigued by the fact that persons who lived in the Balkan countries enjoyed much longer lives than other persons. He felt that it might have something to do with the fact that they consumed large quantities of fermented milk. He later claimed that their prolonged life was due to an organism called Lactobacillus bulgaricus, found in fermented milk which prevented putrefactive

breakdown of protein in the intestinal tract. The type strain with which he worked is now known not to survive in the intestinal tract. However still today many researchers and other people are convinced that there are advantages offered by the consumption of fermented milk products that contain certain Lactobacillus organisms.

Lactobacillus acidophilus has been used for years in the manufacture of acidophilus milk and now some manufacturers include this organism in yogurt cultures. The literature is replete with articles which make claims for acidophilus milk. Another closely related organism, called Bifidobacterium bifidum also plays a protective role in maintaining a proper microbial balance in the intestinal tract. Attempts have been made to use this knowledge to advantage in the livestock industry and this is receiving renewed attention now since Great Britain has moved to ban all antibiotics from livestock feed and the Food and Drug Administration Task Force Report (1972) recently recommended banning of antibiotics in animal feeds in this country. In Europe, it is common practice to feed L. acidophilus to certain farm animals; however, the benefit of this practice when large numbers of viable organisms are not used is questionable. Bengson (1970) emphasized that a microbial balance of a number of species is usually achieved in the healthy individual under normal terrestrial conditions that enables the subject to remain healthy. An imbalance such as occurs in prolonged space flight increases the potential for

disease. The overuse of antibiotics and subsequent elimination of many organisms from intestinal and vaginal flora also provides the opportunity for therapy with concentrates of Lactobacillus organisms.

Scouring or diarrhea in pigs caused by EEC occurs frequently even in the most well-managed swine herds. This has provided the opportunity to explore the possibility of feeding concentrates of Lactobacillus organisms to maintain a proper microbial balance in the intestines in order to reduce the scouring problem. In the present study, concentrates of lactobacilli were fed individually by bottle, as well as through the drinking water system, to test the possibility of mass-feeding of the organisms. During feeding periods numbers of fecal coliforms, lactobacilli, Bacteroides and clostridial organisms were determined as well as the total aerobic and anaerobic bacterial populations. Changes in the flora of different parts of the gastrointestinal tract as well as the incidence of scouring and weight-gain performance also were studied.

The fate of the lactobacilli fed was determined by direct and indirect methods. The indirect method involved isolating lactobacilli from the intestinal homogenate of Lactobacillus MLC-fed pigs and characterizing them using biochemical and fluorescent antibody (FA) staining. Direct evidence of colonization involved the microscopic examination of frozen sections of intestines from Lactobacillus-fed pigs. Fluorescent antibody staining of the tissue also was employed

to get conclusive evidence. The colonization pattern by E. coli as well as lactobacilli also was examined in Lactobacillus-fed pigs which were challenged with EEC.

Since colonization in the intestine by EEC is controlled by a capsular antigen (K88) it was of interest to determine the possible role of antigen make-up in colonization by lactobacilli.

Colostrum is required by most mammals, especially pigs, for postnatal survival. It is believed to inhibit EEC either by means of iron-binding proteins or by immunoglobulins, and in the latter case providing passive immunity to newborn pigs. Attempts therefore were made to examine the effect of colostrum on colonization by E. coli and lactobacilli. In addition, attempts were made to determine the characteristics associated with organisms able to colonize the intestine.

Inhibition of pathogens by lactobacilli was also studied; a possible metabolic inhibitor produced by a Lactobacillus species was isolated and identified and its inhibitory mechanism was studied. Other metabolic end products of the organism which might have some role in inhibition were also studied.

REVIEW OF LITERATURE

Host Resistance to Enteric Infection

Little research attention has been given to determine the role host-determined factors play in the pathogenicity of E. coli and other enteric organisms in the intestines. Factors believed important in this regard are considered below.

Role of Autochtonous Intestinal Bacterial Flora

The growing awareness of the role of intestinal bacteria in health and the urgent need for more definitive knowledge on this subject prompted the organization of an international symposium on the ecology of intestinal flora at the University of Missouri in 1970 and 1972. But interest in this subject is not all recent. As early as 1916, Nissle discussed the possibility that the normal intestinal flora may be a significant factor in the natural resistance of human beings and animals to enteric infections. Direct evidence of a protective role for the normal intestinal flora was presented by Freter (1955 and 1956). He found that oral administration of antibiotics to eliminate components of the intestinal flora of mice and guinea pigs rendered the animals highly susceptible to infection by Shigella and Vibrio sp. Similar work dealing with Salmonella enteritidis infection was reported by Bohnhoff et al. (1954). Miller and Bohnhoff (1963)

subsequently reported that the disappearance of Bacteroides sp. from the intestines was directly related to enhanced susceptibility to salmonella infection. Re-establishment of Bacteroides in the intestinal tract after antibiotic treatment partially restored resistance to infection. Hentges (1969) checked the ability of 15 strains of intestinal bacteria to inhibit Shigella flexnerii. Of the 15 strains tested 14 inhibited Shigella growth in mixed culture. Interactions between members of the intestinal flora and S. flexnerii were also studied in vitro (Hentges, 1967), and it was observed that formic and acetic acids produced by Klebsiella were responsible for inhibiting Shigella sp. According to Gall (1970), "the influence of the balance of the normal flora of the intestinal tract on the health and well-being of the host is well documented. Intestinal bacteria are reported to affect natural resistance (Zubrczki and Spaulding, 1962) and are implicated in metabolism of cancers, serum proteins, cholestrol, hormones, vitamins and incidence of caries (Haenel, 1961)." While a paucity of information is available on the normal bacterial flora of man, Gall (1970) has found that strictly anaerobic bacteria (not facultative anaerobes) comprise over 90% of the total intestinal flora and that symbiotic relationships between different types of organisms present may be important in maintaining the normal balance of microorganisms.

That stress conditions can alter the microbial flora of the

intestinal tract also is now well-documented. This has come to light especially as the effects of bio-isolation on the intestinal flora of astronauts have been noted. Bengson has reviewed this subject (1970), which is regarded so serious as to cause Bengt Gustaffson to state at an ONR-NASA conference on human ecology in space flight (1965, Princeton, New Jersey), ". . . the two most hazardous things an astronaut takes into his capsule on extended flight are his brain and his intestinal flora" (quote from Bengson, 1970). The concern is, therefore, that certain bacteria normally supressed by large number of adventitious organisms, will overgrow and create disease conditions. That this fear is well-founded has been noted by Khazen (1967) who indicated that soviet astronauts experienced functional and structural disturbances along with changes in the composition of the intestinal flora during a 120-day space craft cabin test.

Desirable members of the intestinal flora are not well defined but numerous studies dating from the early 1900s to the present indicate that lactic acid bacteria, especially those of Lactobacillus and Bifidobacteria genera, are important. The precise nature of their beneficial effect, direct or indirect, remains to be proven though studies with breast-fed infants recently carried out by Mata et al. (1972) leave little doubt that intestinal colonization by lactic acid bacteria is a major factor in minimizing enteric disease among newborn infants. The proper balance of organisms in the intestines

of infants also is important to their postnatal adjustment. Attention was drawn to this fact as early as 1899 when Tissier (1899) noted the presence of characteristic Y-shaped (bifidus) lactic acid bacteria in the feces of breast-fed babies to the exclusion of coliforms and other bacteria. Snyder (1940) reviewed the early literature on this subject and noted that the bifidus bacterium (Lactobacillus bifidus, now called Actinomyces bifidus, Rosebury, 1969) was present in 20 out of 21 breast-fed infants, 12 of 19 on supplemented feedings, but in only 12 of 142 weaned infants. It is also well known that in breast-fed infants a stable microflora develops in the colon and feces within 3 or 4 days which usually consists of more than 99% type D IV, Lactobacillus bifidus found nowhere else in the nature (Gyllenberg and Roine, 1957; Gyorgy, 1957; and Haenel, 1970). The fact that the breast-fed babies have a low incidence of colic and other digestive disturbances leads to the conclusion that these organisms are beneficial in infant health. Robinson and Thomson (1952) have reported that infants partially nursed, even for two or three days, showed significantly greater weight gains during the first month than infants completely bottle-fed. They have also reported that bottle-fed infants on formulae supplemented with L. acidophilus showed greater weight gains than control subjects.

Many additional references on this subject exist. The most recent have been reviewed by Brown and Townsley (1970). Of

particular interest, however, is a recent study by Mata and Urrutia (1971) on the intestinal colonization of breast-fed village children belonging to the Maya-Cakchiquel cultural group in Guatemala. This primitive society has not been influenced to any extent by custom and practices of modern technology. The village population is about 1300; the inhabitants live in one- or two-room houses and sleep on floor mats. Mid-wives, who lack knowledge of modern obstetrical practices, assist in the delivery of the infants in homes. Birth is given in the squatting position and therefore fecal contamination of the newly born infants is common. Colostrum and milk from a wet nurse become available immediately and the children are breast-fed for up to four years. Results of this study have shown that half of 30 consecutive newborns had E. coli counts of 10^{10} to 10^{11}/gm of feces during the first 24 hours of life and counts remained at 10^{5} to 10^{11}/gm of feces during the second day of life. Bifidobacteria rarely appeared during the first day of life, but they increased in proportion to age. By the end of the first week, all infants had these bacteria in high numbers (10^{9} to 10^{10}/gm). The frequency of bacteria in 12 breast-fed infants was followed throughout the first year of life; but Enterobacteriaceae, as well as other aerobic and facultative bacteria, were always present in concentrations 2 or 3 logs below the bifidobacteria. Also, of 7,792 weekly cultures, only 40 infections with EEC were found. Infection was the highest when weaning was at its

peak, suggesting that the stress of diet changes may influence intestinal colonization by pathogenic E. coli sero-types. Diarrheal disease caused by Shigella sp. was more significant than that caused by E. coli but here again, the incidence of these organisms was low during the period of exclusive breast feeding. These authors suggested that a probable explanation for the resistance of breast-fed infants to intestinal colonization with enteric pathogens is the predominence of the bifidus flora which is stimulated by maternal milk.

An explanation for the predominence of bifid bacteria in the feces of breast-fed infants has been sought. Workers at the University of Pennsylvania identified the bifidus factor present in human milk as a number of oligosaccharides containing glucose, galactose, fucose and N-acetylglucosamine (Gyorgy et al., 1952). These findings were confirmed by Lambert and Zilliken (1965). According to Rosebury (1969), however, it is doubtful that the presence of the bifidus factor in human milk can account for the predominence of A. bifidus in the nurseling stool since the bacterium studied by the Pennsylvania group is not the characteristic strain found in the feces. Data of Gyllenberg and Roine (1957) suggest that the role of human milk is to inhibit growth of other bacteria to allow dominance by A. bifidus. More recently Yoshioka et al. (1968) observed nutritional differences between strains of bifid bacteria isolated from infants and suggested that carrot extract used in treatment of infant diarrhea

(Kuromiya, 1960), contained growth factors for A. bifidus in addition to that reported by Kanao et al. (1965). These latter workers identified a new bifidus factor as the coenzyme A precursor, pantethiene phosphate.

The exact source of the bifid bacteria that gain entry into the nurseling infants remains a mystery today (Rosebury, 1962 and 1969). Shirota (1962) has suggested that they are derived from the Döderlein bacillus (Döderlein, 1892), a beneficial bacterium inhabiting the human vagina. This, however, has not been established. Nevertheless, the vagina, like the gastrointestinal tract, depends on dominance by lactic acid bacteria to supress undesirable types which may cause vaginitis.

Role of Colostrum and Human Milk

Pediatricians hold a common opinion that breast-fed infants experience less infectious disease than those artificially fed. There are many field observations indicating the important role breast-feeding plays in allowing the infant to resist enteric infections. The mechanisms have not been soundly established; but several factors appear to be involved, such as specific antibodies to infectious agents, factors stimulating or inhibiting certain intestinal micro-organisms or presence of non-specific antimicrobial substances.

Immunoglobulins in Human Milk

Schlossman and Moro (1903) pioneered the demonstration of a relationship between proteins in blood serum and milk. For example, the blood immunoglobulin fractions IgA, IgG, IgM, and IgD are all present in human milk. Since breast milk antibodies are not reabsorbed by the infant (Vahlquist, 1958), their protective role has been brought into doubt. The dominating immunoglobulin of milk, as well as of other secretions, is secretory IgA (Tomasi et al., 1968). Ammann and Steihm (1966) showed that IgA is present in initial colostrum to the extent of 17 mg/ml and in a four-day colostrum at 1 mg/ml; these amounts exceed those of IgG and IgM. The concentrations of IgA in bovine colostrum is less than in the serum (Murphy et al., 1964). All immunoglobulins have antibody activity and serum IgA contains antibodies of all types (Kriebel et al., 1969).

According to recent concepts (Tomasi and Bienenstock, 1968), IgA is a mediator of local immunity. Therefore, a protective role of milk antibodies in humans should be sought on the mucous membranes of the gastrointestinal tract. In agreement with such a local function is the observation that colostrum antibodies protect against gastrointestinal infections (Mata and Urrutia, 1971; Svirky-Gross, 1958). Furthermore, a recent study has shown that breast milk consumption was significant for protection against neonatal

septicaemia (Winberg and Wessner, 1971). Gindrat et al. (1972), in a preliminary study showed antibodies against the 'O' antigen of some of the most common serotypes of E. coli to be present in human milk, usually in high titers. Those infants who had consumed significant amounts of breast milk with high titers of antibodies against E. coli, had such antibodies in their stools. The possibility that these antibodies, which presumably were of the secretory IgA type, may protect the gastrointestinal mucosa of the neonatal against microbial invasion should be further investigated.

Stimulation of Intestinal Microorganisms

Early in the history of medical microbiology, Tissier (1899) isolated from the feces of breast-fed infants a Gram-positive, non-motile, anaerobic bacillus Bacterium bifidum which dominated all other species. A principal in human milk (bifidus factor) promotes development of this characteristic microflora. Gyorgy (1953) added breast milk to a culture medium rendering it capable of supporting the growth of an organism called Bifidobacterium bifidus var. Pennsylvanicus. Highly active concentrates of the bifidus factor on hydrolysis produced N-acetyl-D-glucosamine, L-fucose and D-galactose.

Bifidobacteria metabolize a variety of sugars, producing large amounts of acetic and lactic acids and trace amounts of formic and

succinic acids (Moore et al., 1970). These products are responsible for the low pH of feces of wholly breast-fed infants (Mata and Wyatt, 1971). Gyllenberg and Roine (1957) found bifidobacteria to represent as much as 99% of the fecal flora of breast-fed infants, in contrast to less than 60% for infants receiving boiled human milk or cow's milk.

That a human milk diet leads to a bifidobacteria type flora is certain, although mechanisms remain unclear. The intestinal tract of wholly breast-fed infants is resistant to infection by pathogens such as Shigella and intestinal protozoa as reported by Mata and Urrutia (1971). Bullen and Willis (1971) suggested that the predominance of L. bifidus, which is associated with a low pH in the feces of breast-fed infants, may be related to the supression of E. coli in the intestine. Mata and Urrutia (1971) have stated that human milk has inherent positive anti-infective properties, such as the intestinal guardian function of the flora in particular.

Non-specific Factors

Masson and Heremans (1971) have shown the presence of large quantities of iron-binding protein of which the greater proportion was lactoferrin, though small amounts of transferrin were also present. Bullen et al. (1972) have shown that lactoferrin in combination with specific antibody to E. coli is responsible for the bacteriostatic

effect. They have also suggested that the iron-binding proteins of milk play an important part in resistance to E. coli infection, particularly in the small intestine. Several other workers have demonstrated the bacteriostatic effect of iron-binding proteins (Masson and Heremans, 1966; Oram and Reiter, 1968).

Pathogenicity of EEC

Neter et al. (1955) introduced the term enteropathogenic E. coli (EEC) to distinguish those strains of the organism capable of causing enteric infections from those associated with other disease. While first described by Adam (1923), EEC is recognized today as a serious problem in hospitals (Tomic-karovic and Fanjek, 1962; South, 1971; and Gorbach and Khurana, 1972), where it causes severe and highly fatal infantile gastroenteritis. It is also frequently involved in hospital acquired infections of different types. One recent study (Gardner and Carles, 1972) indicated that 43% of all urinary tract infections acquired during confinement were caused by E. coli. A problem of some magnitude seems to exist with laboratory animals, too, judging from the report of Schiff et al. (1972); in this study, EEC strains were isolated from mice, rats, hamsters, gerbils, guinea-pigs, agouti, beagle dogs, and rhesus monkeys, only 25% of which showed disease symptoms.

Escherichia coli Infections in Swine

Human infections caused by enteropathogenic strains of E. coli is paralleled by a strikingly similar disease problem in pigs, recently reviewed by Kohler (1972) and Barnum (1971). A number of research workers have examined the causes of coliform-associated piglet mortality and in nearly every instance hemolytic strains of E. coli figured predominantly in death caused by enteritis and scouring. Kennworthy and Crabb (1963) noted that the hemolytic E. coli appeared in the jejenum and ileum of baby pigs at the onset of diarrhea and gastroenteritis. Other bacteria did not increase when this occurred. The intestinal flora associated with enteritis of early-weaned pigs was studied by Chopra et al. (1963). These workers found the greatest increase in E. coli (coliforms) when diarrhea occurred. They also indicated that there was a balance between lactobacilli and coliform bacteria in non-scouring pigs which became greatly altered when diarrhea was present. Hill and Kennworthy (1969) implied that high levels of hemolytic E. coli may be tolerated in the presence of established and continuously-proliferating lactobacilli.

Dillard (1964) pointed out that colibacillosis (baby pig scouring or diarrhea caused by E. coli) is a major cause of economic loss in the swine industry. Even well-managed herds, according to

McErlean (1960), often are involved and the contents of the small intestine usually provides an apparently pure culture of E. coli, many other reports provide over-whelming evidence that this bacterium is the chief cause of piglet death and impairment of herd performance. Coalson and Leece (1973) reported a 20 to 30% mortality in pigs reared under conventional methods as opposed to no mortality in pigs taken away from the sow immediately after birth and reared under extremely clean conditions. Recently Glantz and Kradel (1971) noted colibacillosis in swine in the United States caused by the Abbotstown strain of E. coli. This serotype was found by workers in England and Canada to produce a powerful enterotoxin.

Most strains of E. coli causing diarrhea in pigs produce hemolysin while a few non-pathogenic strains inhabiting the alimentary tract of man and animals have this property (Smith, 1963). Studies by Smith and Hall (1967a and 1967b), Kohler (1971), and Kohler and Cross (1971) have shown that enterotoxin present in spent cultures and in whole cell lysates of EEC strains would produce positive dilatation reactions in ligated sections of pig intestine as well as diarrhea and frequently death when fed to gnotobiotic pigs.

The scouring or diarrhea that occur in newborn pigs infected with E. coli is accompanied by a shift in the proportion of bacteria normally found in the intestinal tract. Kennworthy and Crabb (1963)

found that while the tract is sterile at birth, within 24 hours lactobacilli, Gram-positive cocci, Clostridium perfringens and E. coli appear; Gram-negative cocci and Bacteroides appeared later. When scouring occurred, hemolytic strains of E. coli increased by at least 99% and stress brought on by weaning contributed to the decrease and shifting balance of organisms. Other workers also have emphasized the importance of a proper microbial balance in the intestinal tract to disease resistance in pigs (Cole et al., 1968; and Kershaw et al., 1966). This naturally has led to studies in which certain bacteria, especially lactobacilli, have been fed to scouring pigs infected with E. coli in an attempt to restore the proper balance of bacteria.

Moon and Whipp (1971) indicated that the characteristics that enable EEC to colonize the intestinal tract are unknown but that the enteropathogenic potential was made up of two components--first, the colonization itself in the jejenum in large numbers, and second, the stimulation of movement of water and electrolytes across an intact intestinal epithelium into the lumen. Bertschinger et al. (1972) recently indicated that human EEC can be divided into two groups--one that penetrates the intestinal epithelium and does not produce enterotoxin, and a second that remains in the lumen (colonizes) and produces enterotoxin.

The enterotoxin produced by EEC causes acute accumulation of fluid in ileal loops of pigs (Smith and Halls, 1967a; Gyles and

Barnum, 1969; Moon and Whipp, 1971) in a manner comparable to enterotoxin from Vibrio cholerae. In fact, Smith and Sack (1973) recently showed that the enterotoxins from EEC and V. cholerae were antigenically related; cholera antitoxin from immune sera neutralized E. coli toxin in rabbit ileal loops, but the reverse was not true. A number of studies on the nature of EEC enterotoxin have been made (Smith and Halls, 1968a and 1968b; Kohler, 1968; Gyles and Barnum, 1969; Smith and Gyles, 1970; Kohler, 1971; and Jacks et al., 1973) and two forms of the toxin are known. One is heat-labile, non-dialyzable, antigenic and rapidly inactivated at pH 3.0; the other is heat-stable, slowly dialyzable, non-antigenic and unaffected by pH levels from 1.0 to 10.0. Both forms of enterotoxin are controlled by transmissible plasmids (Gyles, 1972).

It has been suggested by a number of workers (Smith and Halls, 1967a; Kohler, 1968; Gyles and Barnum, 1969; Smith and Gyles, 1970; and Kohler, 1971) that enterotoxin from EEC strains colonizing the intestine is the primary agent responsible for diarrhea (colibacillosis) in piglets. Steevens et al. (1972) recently reported that pigs were highly sensitive to enterotoxin-induced diarrhea when dosed orally during the first three days of life; sensitivity gradually decreased up to 38 days of age. Pigs weaned at 38 days for 3 days were resensitized while the non-weaned animals remained passive.

Attempts to establish enteric infections in normal experimental

animals have not always been successful unless antibacterial drugs were administrated to supress the indigenous intestinal flora (Mushin and Dubos, 1965). In 1967, Drucker et al. (1967) demonstrated the colonization of the intestinal epithelium of ligated segments of rabbit small intestine by human EEC strains. The next year, Smith and Halls (1968a) reported, as evidence of colonization, the recovery from wall scrapings of pig small intestine greater numbers of EEC than could be found in the contents. The pathology of attachment to and penetration of the ileum of newborn pigs as observed by light and electron microscopy of thin sections was then described by Staley et al. (1969). Using fluorescent antibody staining of cryostat section of the intestine derived from pigs exposed to EEC, Arbuckle (1970) demonstrated the adherence of three strains of EEC to villi of the small intestine; non-EEC were found in the villi in only small numbers. While the FA technique did not demonstrate the presence of EEC in the mucopolysaccharide layer lining the intestine, a follow up study (Arbuckle, 1971) demonstrated the colonization of the mucin layer as well as the villi. Two other papers on the microscopy of colonization by EEC have also been published--one an FA study (Drees and Waxler, 1970a) and the other, an electron microscope study (Drees and Waxler, 1970b).

Research attention also has been given to the role of plasmids in pathogenicity of E. coli. A plasmid controls the production of

alpha-hemolysin production by E. coli (Smith and Halls, 1967a), and it has been noted that most strains producing diarrhea in pigs are hemolytic (Sojka, 1965). However, alpha-hemolysin may not be a virulence determinant since the diarrhea-producing capacity of EEC was shown by Smith and Linggood (1971a) to be independent of the presence of this plasmid. Colicins also do not seem to have any effect on virulence of EEC (Craven and Barnum, 1971). Drug resistance plasmids apparently have not been studied in EEC as virulence attentuation or potentiation factors, but both the K88 antigen and enterotoxin plasmids have been shown to be important in virulence (Gyles, 1972; Smith and Halls, 1967a; Smith and Halls, (1968b). Pili also may be plasmid-mediated virulence determinants (Gyles, 1972).

Vaccination Against E. coli Infection

Gordon and Luke (1958) stated that vaccination of sows with autogenous E. coli vaccine reduced diarrhea and mortality in piglets. On the other hand, Jones et al. (1962) reported that vaccination of sows had no apparent effect on the incidence of diarrhea. In none of the above experiments, however, were the pigs exposed to a definite challenge by a strain of EEC. Therefore, Rutter and Anderson (1971) challenged animals with EEC and observed that although there was reduction in mortality of pigs from vaccinated dams compared

with non-vaccinated dams, the reduction was not significant when the variation between litters was taken into account. However, bacteriological observation suggested that an anti-bacterial factor impaired the ability of EEC to become established in the intestinal tract of piglets from vaccinated dams. In studies on experimental E. coli diarrhea in gnotobiotic piglets, Kohler and Bohl (1966), Kohler (1967), and Miniats et al. (1970) observed that the protective effect of antiserum was not associated with a marked reduction in the concentration of organisms of the infecting strain in the contents of the small intestine, suggesting that this effect was anti-enterotoxic rather than anti-bacterial in nature. On the other hand, in somewhat similar studies on conventionally-reared, weaned pigs, Smith and Linggood (1971b) found much lower concentrations of the infecting organisms in the small intestine of antiserum-protected animals than in unprotected animals. This suggested that the anti-bacterial effect of antiserum was important in preventing clinical infection. In an attempt to clarify this controversy, Smith (1972) reported from his experiments that after oral administration of antisera and E. coli, only a low concentration of EEC organisms was found in contents of the small intestine of piglets in which diarrhea was controlled by antiserum; this indicated that anti-bacterial antibody was important. In an excellent review on the control of neonatal colibacillosis of swine, Barnum (1971) deals mainly with the host and methods of

enhancing its immunity to E. coli and pathogenic factors associated with the organism.

Immunity gained in pigs as a result of feeding or injecting different vaccine preparations has also been studied recently (Kohler and Cross, 1971; Svendsen, 1971). Rejnek et al. (1968) reported that feeding colostrum or serum from immunized sows would protect gnotobiotic pigs from pathogenic E. coli. Svendsen (1971) and Svendsen et al. (1971) confirmed these findings and noted further that colostrum from intra-mammary vaccinated sows appeared to give more protection to newborn pigs than those materials from intramuscular inoculated sows. As a result of these and similar studies, some hope is now held for development of specific strain vaccines which would be used under field conditions to immunize swine. Similar studies in humans apparently have not been conducted. Shreeve and Thomlinson (1970 and 1971a) showed that piglets may be hypersensitive to certain serotypes of E. coli at birth, and that pregnant sows immunized with either hen's egg albumin or E. coli extracts conferred hypersensitivity to their young, whether or not they had received colostrum. Sensitization of piglets may have resulted from the transfer of maternal antibody across the placental barrier or from the transfer of antigen, thus leading to active immunization of the piglets. Shreeve and Thomlinson (1971b) also showed that immunizing the pregnant sows result in utero-sensitization of piglets.

Wilson (1972) in his elaborate studies showed that milk and colostrum from sows vaccinated with a live formalin-treated vaccine afford protection against experimentally induced E. coli enteritis. He also showed that immunoglobulins ingested (3 gm/day) were more effective than circulatory antibodies against E. coli enteritis. The IgG immunoglobulins contained anti-enterotoxins, multiplication inhibition factors and the ability to protect against experimental E. coli enteritis when given orally.

Lactobacilli and Intestinal Disease Therapy

Attempts to manage the bacterial flora of the human vagina to improve health, now being practiced by some gynecologists, have been preceded by many efforts to alter or maintain a proper microbial balance in the intestinal tract for the same reason. Since the early work of Metchnikoff (1903, 1907, and 1908) and the clarification provided by Rahe (1915) that L. acidophilus rather than L. bulgaricus implanted in the intestinal tract, lactobacilli have been used extensively in therapy. The older literature regarding its therapeutic value has been documented in one bibliography of abstracts (Frost and Hankinson, 1931) and three textbooks (Kopeloff, 1926; Rettger and Cheplin, 1921; Rettger et al., 1935).

With the introduction of antibiotics taken orally as therapy against systemic infections of various types, patients began to

complain of discomfort in the gastrointestinal tract. Yeast and mold infections were often diagnosed in these cases (Huppert et al., 1953) and standard therapy has been the use of concentrates of L. acidophilus (Duggan et al., 1957; Gordon et al., 1957; Rafsky and Rafsky, 1955; Winkelstein, 1956). The ability of this organism to produce anti-bacterial substance (s) has also been noted (Gordon et al., 1957; Polonskya, 1952; Vakil and Shahani, 1965), the most complete study being reported by Vincent et al. (1959). Furthermore, dried preparations of the organism are available in pharmacies for use in establishment and maintenance of L. acidophilus in the intestines; most are ineffective, however, because low numbers of viable organisms are present.

Studies in radiation biology with whole animals also have emphasized the importance of a balanced intestinal microbial flora in healthy animals. In this regard, Vincent et al. (1955) substantiated earlier reports that lactobacilli constitute the predominant gut flora of small laboratory animals. They went on to show that post-irradiation bacteremia caused by coliform and pseudomonads results when lactobacilli decline in the small intestine of rats.

Recent reports by Dubos et al. (1965), Savage and Dubos (1967), Savage et al. (1968), and Savage (1970) have documented the intimate association between anaerobic streptococci, lactobacilli, fusiform bacteria and yeasts in particular areas of the epithelium of the

gastrointestinal tract of mice. These findings again emphasize the importance of a balanced population of microorganisms in healthy animals. That feed plays an important role in this balance was emphasized when these workers (Savage and Dubos, 1967) found that yeast appear and establish colonies in the mucin of secreting epithelium only after the animals are weaned. In a subsequent report, Savage (1969) found that administrating penicillin to rats and mice resulted in a replacement of lactobacilli by Torula yeasts, a situation which persisted as long as antibiotic was administrated; when penicillin treatment was discontinued, indigenous lactobacilli again colonized the epithelium.

Recent literature also attest to the value of using lactobacilli in treatment of intestinal disorders, though one report (Mocquot and Hurel, 1970) questions their value. Hawley et al. (1959) have reviewed factors important in successful implantations of lactobacilli in the human. At least two considerations are important; that large numbers of viable cells be fed and that a fermentable carbohydrate be available to the cells in the intestinal tract. Beck and Nechels (1961) used lyophilized cultures of L. acidophilus to treat 59 patients with different types of diarrhea, constipation, abnormal fermentations, and food poisoning. Of these, 22 were cases of diarrhea caused by antibiotic treatment; 20 excellent and 2 good therapeutic results were obtained in 17 cases and fair results

in two cases. Failure occurred in one case of colostomy with diarrhea. Macbeth et al. (1965) used L. acidophilus in the treatment of systemic encephalopathy, a toxic condition believed caused by bacterial degradation of nitrogenous substances in the lower intestine and absorption of toxic materials, especially ammonia and amines. Treatment of this disease presently involves evacuation of the flora of the gut, and surgical removal of the colon, all designed to interrupt the activities of bacteria high in urease and amino acid oxidase activity. In their studies, Macbeth and colleagues (1965) altered the intestinal flora of two hepatic encephalopathy patients by feeding L. acidophilus. Urease and amino acid oxidase activities as well as blood ammonia levels were lowered in both patients when aerobic microbial flora was supressed. These findings were generally confirmed by Reed et al. (1966) and Muting et al. (1968).

L. acidophilus therapy in man and domestic animals is more widely practiced in other countries, especially Europe than in this country (Bryan, 1965; Cattan and Milstein, 1960; Kuemmerle, 1958; Lachner and Bieler, 1961; Low, 1959; Mironenko, 1958; Siegenthaler et al., 1965; Skorodumova, 1959; Teply, 1961; Tomic-karovic, 1963; Tomic-karovic et al., 1961; Tomic-karovic et al., 1964; Vicek and Kneifl, 1964). It is note-worthy that Tomic-karovic and Fajnek (1962) demonstrated the effectiveness of L. acidophilus milk in destroying pathogenic E. coli, in vitro and in vivo. The in vivo

effect was tested on 20 infants suffering from diarrhea caused by E. coli serotype $O_{111}B_4$. In all cases, the E. coli which was resistant to a number of antibiotics, disappeared from the stools of the infants within 1 to 5 days of starting the therapy with acidophilus milk; the infants made a rapid recovery. Comparable results were obtained by Aritaki and Ishikawa (1962), Fedetov et al. (1966), and Pene et al. (1966). In addition, Vicek and Kneifl (1964) aided the postnatal adjustment of premature infants by twice daily administration of capsules of "Omniflora," mixed culture of L. acidophilus, A. bifidus, and a non-pathogenic E. coli. These organisms became established in the intestines of 20 out of 24 premature infants within one week.

Japanese scientists have been intensely active in studying the relationship between the human intestinal flora and disease, especially since 1936 when Shirota (1962) isolated the Yakult Lactobacillus which would implant in the gastrointestinal tract. A summary of these research reports has recently been prepared in English (1971). A large industry with franchised plants in Japan, Taiwan, Hong Kong and Brazil now provide Yakult as health food to millions of people daily. In Japan alone over 15 million bottles (65 ml capacity) are consumed each day. The product is a pleasant-tasting, slightly sour, thin liquid containing an extract of Chlorella algae, added vitamin C and greater than 10^8 viable cells of the Shirota Lactobacillus strain

per ml (Ozawa and Joo, 1970; Yakult, 1971).

Just as infant coliform diarrhea has a parallel disease in swine (colibacillosis), a disease of piglets similar to encephalopathy is common. This is to be expected since, from the above cited references, it is clear that the microbial balance in the intestinal tract is the underlying cause in each case. Porter and Kennworthy (1969) have studied colibacillosis in pigs and cited other pertinent references. In fact, the positive response to antibiotics in swine feed has been suggested (Larson and Hill, 1960) to be caused by inhibition of amine forming bacteria such as E. coli in the intestinal tract. Furthermore, Hill et al. (1970) succeeded in reducing the intestinal amine level and scouring in weaned pigs by feeding acidophilus milk supplements.

Feeding lactic acid bacteria, especially L. acidophilus to swine as therapy by restoring a healthful microbial balance has been the subject of a few reports. Mollgaard (1946) showed that the presence of lactic acid facilitated absorption of calcium and that pigs fed cultures of lactobacilli had increased amounts of lactic acid in the intestines. These pigs grew better than the control group which had not been given lactobacilli. These effects have been confirmed (Cole et al., 1968; Kershaw et al., 1966) with a suppression of E. coli also noted in each case. Also, Leitgeb (1961) observed that in growing, fattening pigs, the count of E. coli in the intestinal tract was

inversely related to that of lactic acid-producing bacteria, chiefly L. bifidus. Pasicynj (1959), Redmond and Moore (1965), and Nedyalkov et al. (1967) also have noted a beneficial effect on swine in terms of weight gain and reduced enteritis by feeding L. acidophilus. In Sweden, a commercial preparation called "Majrdes" is used for prophylaxis and therapy of intestinal disturbances in pigs. Olsson (1961) fed 50 gm of this lyophilized L. acidophilus preparation daily (10 days) to counteract diarrhea in weaned pigs. Enteritis disappeared and weights of the animals increased 25 to 40%. Also, at least two patents (Peer, 1967; Wenner-gren Medical Laboratory, 1968) have been issued on methods to prepare lactic acid bacteria to use in animal feed supplements. Furthermore, a product manufactured in France (Biacidol-Laboratorie de Biolgoie Industrielle Appliquee, Saint-quen) consisting of lyophilized L. acidophilus is widely used in France, Denmark, and Germany to treat domestic animals suffering from intestinal disorders. Recently, a product was patented (U. S. Pat. 3,713,836, 1973) which contains a strain of Streptococcus fecium grown in an aqueous nutrient medium under anaerobic conditions. The resulting bulk of bacteria was freeze dried and when administrated to domestic animals was capable of controlling the bacterial flora in the intestines, resulting in increased growth. A similar preparation of S. fecium (Rumapo SF 68) is widely used in Netherlands for protecting against enteric infections

(Ruitenberg, 1973). Mutai et al. (1971), describing the characteristic features of a beverage containing live lactobacilli, proposed that the Lactobacillus used must be an enteric type, of human origin, able to multiply in the intestine; it also must be non-pathogenic and capable of producing lactic acid and be resistant to its own metabolic products.

Sandine et al. (1973) in their detailed review of literature, discussed the role played by lactic acid bacteria in human and animal health; antibiotic therapy was also condsidered with regard to public health.

METHODS AND MATERIALS

Taxonomy of *Lactobacillus* Organism Used

Culture Used

Frozen concentrates of the *Lactobacillus* organism used in the study were obtained from Microlife Technics, Sarasota, Florida. The bacterium was originally isolated from the human intestinal tract by Mr. Stewart M. Farr. The concentrate was plated on the medium of Rogosa *et al.* (1951) and a single colony isolate (MLC strain) was characterized using the tests indicated in the report of the *Taxonomic Sub-Committee on Lactobacilli and Closely Related Organisms* (Anonymous, 1968). The enzymatic method of Mattsson (1965) was used to determine the type of lactic acid isomer produced. The biochemical characters of the organism were determined using the API test pack system. The test sets, which consist of sterile prepared media in plastic impress forms, were obtained from Analytab Products, Inc., 516 Mineola Ave., Carle Place, N. Y.

Guanine Plus Cytosine Content

DNA extraction was carried out using a modified procedure of Marmur (1961). As it was difficult to lyse the organism, the dual enzyme system of lytic factor (5% v/v) plus lysozyme (2 to 4 mg/ml-Sigma Chemical Company) as described by Sriranganathan *et al.*

(1973) was used. The suspension was incubated at 37 C and checked for complete lysis every 30 minutes by a spot test (one drop of cell suspension plus one drop of 25% sodium lauryl sulfate). Complete lysis was indicated by clearing and increasing viscocity. Deproteinizations (initial and after ribonuclease treatment) were carried out with saline-EDTA-equilibrated liquid phenol at pH 7 to 8. Other deproteinizations (a minimum of four) were carried out with Sevag's solution (chloroform:isoamylalcohol:24:1).

The DNA base composition was determined using thermal melting (Tm) data and the equation of Mandel et al. (1970). E. coli K12 DNA was included in each melting as an internal standard.

DNA-DNA Hybridization

To confirm identification of the organism, DNA-DNA hybridization was conducted as described by Sriranganathan et al. (1973). Tritium labelled DNA from L. lactis ATCC 12315 was used as the reference DNA.

Serological Studies

To determine the antigenic relation of the MLC Lactobacillus strain to other lactobacilli, the Öuchterlony immunodiffusion technique was employed. Organism used in the study were obtained from the stock culture collection maintained in the Department of

Microbiology, Oregon State University. The following organisms were used in the study:

Microlife concentrate strain Lactobacillus (MLC)

Lactobacillus FH obtained from the Corvallis Farm Home Swine Center

Lactobacillus acidophilus Farr

Lactobacillus lactis ATCC 12315

Lactobacillus bulgaricus ATCC 11842

Lactobacillus salivarius ATCC 11742

Lactobacillus casei C-17

Lactobacillus plantarum ATCC 4917

Lactobacillus jensenii ATCC 25258

Preparation of Soluble Antigen

The organisms were grown in 500 ml quantities of MRS broth at 37 C for 24 to 36 hours. The cells were harvested, washed ten times in normal saline and then resuspended in a phosphate buffered saline solution at pH 7.0. To obtain a standard concentration of antigen from all the organisms, the suspensions were adjusted to an O. D. reading of 1.0 using a Bausch and Lomb Spectronic 20 spectrophotometer. The standard suspensions were then sonicated at 0 C using a model W-185 Sonifier cell-disruptor. A smear of the suspension was frequently made and checked for intact cells. The suspensions were sonicated until few intact cells remained. To

prevent contaminations among the suspensions, the disruptor horn was thoroughly washed after each sonication. After sonicating, the suspensions were centrifuged at 5000 rpm for 10 minutes and the clear supernatant obtained was used as the soluble antigen.

Production of Antisera

Antiserum was prepared only against Lactobacillus MLC. The procedures used will be described elsewhere.

Ouchterlony Test Procedure

A special 1.4% noble agar (Difco) was prepared in phosphate-buffered saline (pH 7.0) and 5 ml of the melted agar poured into small petri dishes (60 X 20 mm). After the agar was solidified, fine wells of 6 mm in diameter were made in the agar. Four of the wells were equally spaced from one another in a ring 12 mm from the center well. Two wells were filled with the MLC strain antigen and the other two wells were filled with antigen from one of the lactobacilli listed above. The central well was filled with antisera against the MLC Lactobacillus. The plates were incubated at 37 C in a humid atmosphere for 72 hours and examined.

Normal Fecal Bacterial Flora

Pigs of the following age groups were used in the study: one week, three weeks, weaned (six weeks) and adult pigs. Fecal

samples were collected with a sterile swab, taking care to prevent contamination from the perineal region. Bacterial counts were made according to the method described by Smith and Crabb (1961). Within 30 minutes of collection, a 1:10 dilution (w/v) of the fecal sample in distilled water was made and mixed well using a Vortex mixer to yield a homogenous suspension. Occasionally it was not possible to obtain a full one-gram sample from young pigs and in such cases, the (w/v) dilution was increased from 1:10 to 1:100. A summary of the techniques used to obtain viable counts of the fecal bacterial flora of pigs is given in Table 1.

The principal bacteria identified and counted were Escherichia coli, Clostridium perfringens, streptococci, lactobacilli, Bacteroides sp. and Staphylococcus aureus. E. coli counts were estimated from the number of typical lactose-fermenting colonies on MacConkey's agar. Typical colonies were further characterized using the various biochemical tests described in Bergey's Manual of Determinative Bacteriology (1969).

Lactobacillus organisms counted were based on characteristic colonies found on Rogosa's agar (Rogosa et al., 1951). A typical colony was picked and characterized using the biochemical tests provided in the API test pack system as well as the methods described by the Taxonomic Sub-Committee on Lactobacilli and Closely Related Organisms (Anonymous, 1968). Usually only one colony type was

Table 1. Techniques Used to Obtain Counts of the Viable Fecal Bacterial Flora of Pigs.

Organism	Differential Method
E. coli (coliforms)	MacConkey's agar (Difco) incubated aerobically at 37 C for 24 hours.
Lactobacillus sp.	Rogosa's agar incubated in presence of 95% nitrogen and 5% CO_2 at 37 C for 48 hours.[a]
Clostridium sp.	Neomycin blood agar[b] incubated in presence of 95% nitrogen and 5% CO_2 at 37 C for 24 hours.
Streptococci (enterococci)	Streptococcus fecalis (SF) medium (Difco) incubated in presence of 95% N_2 and 5% CO_2 at 37 C for 48 hours.
Staphylococcus aureus	Vogel-Johnson agar (Difco) incubated aerobically at 37 C for 48 hours.
Bacteroides sp.	Neomycin blood agar[b] incubated in presence of 95% N_2 and 5% CO_2 for 48 hours.

[a] To achieve the N_2-CO_2 atmosphere, Torbal steel cylinders (Model AJ-2) containing the inoculated plates were evacuated and filled three times with the gas mixture delivered from a pressure tank.

[b] 70 μg/ml of neomycin sulfate was used in neomycin blood agar.

evident; however, occasionally different colony types were seen which also belonged to the genus Lactobacillus.

Bacteroides organisms enumerated were non-hemolytic colonies appearing on neomycin blood agar grown under anaerobic conditions. Typical colonies were further characterized using the tests called for in Bergey's Manual of Determinative Bacteriology (1969). Hemolytic, punctured-type colonies were found to be clostridia and were further characterized as Clostridium perfringens using the methods of Bergey's Manual of Determinative Bacteriology (1969). Streptococcal counts were made on SF agar and colony isolates were further characterized as Streptococcus fecalis. Total aerobic counts were obtained using blood agar incubated aerobically at 37 C for 48 hours and total anaerobic counts on the same medium incubated anaerobically at 37 C for 48 hours.

Feeding Experiments

Bottle Feeding

A litter of nine pigs was divided into two groups and fecal samples were taken from each pig before putting on test. The plating procedure was the same as described earlier. Attention was given to E. coli, Lactobacillus, total aerobic and total anaerobic counts. Thus, samples were plated on MacConkey's, Rogosa's and two sets of blood agars (one incubated aerobically and the other

incubated anaerobically). One group of four pigs from this litter was fed the usual ration while the ration of the other five pigs was supplemented with the concentrate of MLC Lactobacillus. The feeding schedule for the concentrate was as follows:

10 to 15 ml per pig per day from birth to weaning

30 to 40 ml per pig per day after weaning

Concentrate feeding was started when the pigs were three days old. Fecal samples were taken every day for the first week, every other day from the second week until weaning and every third day thereafter. The sampling and plating was done as described earlier.

A second experiment was undertaken with a different feeding schedule. A litter of nine pigs was used as above with the daily feeding schedule as follows:

0 to 15 days of age . . . . . 10 ml per pig

16 to 30 days of age . . . . . 15 ml per pig

31 to 8 weeks of age . . . . . 30 ml per pig

Over 8 weeks of age . . . . . 50 ml per pig

Fecal samples were plated in the same manner as before. A record of scouring for the above pigs was also maintained.

Feeding Through the Drinking Water System

After studying the influence of the Lactobacillus MLC concentrate on pigs fed individually, the concentrate was fed to a large

number of pigs through the drinking system to determine the feasibility of feeding animals on a large scale. Twenty-four pens of pigs (12 control and 12 experimental) were studied. The water supply of the 12 experimental pens was fitted with a commercial water proportioner (Fig. 1), which metered Lactobacillus concentrate into the water at a fixed rate (1 oz/gal) as the water was consumed by pigs. The concentrate to be distributed into the water system contained 10% lactose and was stored in a four-liter bottle in a refrigerator next to the proportioner. Fresh bacterial concentrate and lactose were added daily and more often as needed when water consumption increased due to high air temperatures, etc. Attempts were made to keep the daily concentrate supply constant and also to minimize the waste.

The viability of the MLC concentrate organisms in water with added lactose was studied before beginning the herd feeding experiment. Organisms passing through the water distribution system were also studied for viability after the concentrate was added by plating a water sample at the drinking source. This sample was compared with a concentrate sample taken from the four-liter bottle at various time intervals. In addition, the number of organisms which individual pigs were consuming each day was approximated by determining the dilution factor of the concentrate in the water system and the approximate amount of water individual pigs consumed

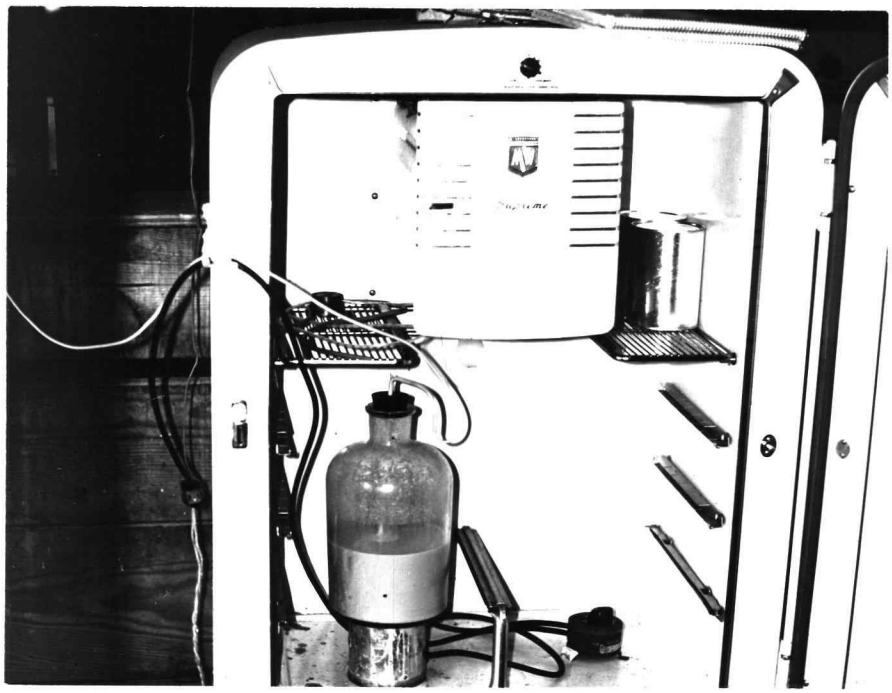

Figure 1. Photograph of Concentrate Reservoir which Supplied Lactobacillus lactis MLC Concentrate to the Water Proportioner.

each day. The Lactobacillus MLC concentrate was at about 50 X 10^9 viable cells/ml; 900 ml were added to 2980 ml of water containing about 10% lactose which provided a dilution factor of 900/3880 or 0.23. Therefore there were (50 X 10^9)(0.23) or 11.5 X 10^9 cells/ml in the water proportioner reservoir. Since the proportioner delivered one ounce (30 ml) per gallon (3880 ml) a second dilution factor of 30/3910 or 0.008 resulted. Therefore, there were (11.5 X 0.008) or 0.092 X 10^9 cells/ml in the water lines. This assured that pigs drank water containing at least ten million cells/ml, since it already was established that the cells were quite stable with regard to viability.

Both the control and experimental pigs received the same feed. The pigs studied were brought into the experimental and control pens at about one week of age and were managed similarly. As in the previous experiment, fecal samples were taken from each litter before they received the Lactobacillus containing diet. Random samples were taken from three pigs in each pen including the sow, if present, once each week for a three-month period. The plate counts were made in the same way as described for the earlier experiment.

Effect of Feeding MLC Concentrate on the Bacterial Flora of Intestinal Parts

Animals were grouped as follows for the feeding experiment:

Control, non-scouring, 3 weeks of age . . . Group I

Control, scouring, 3 weeks of age . . . . . Group II

Lactobacillus-fed group which received the MLC concentrate for 17 days . . . . . Group III

The parts of the gastrointestinal tract examined were the stomach, proximal small intestine (first four inches) and distal small intestine (second twelve inches); fecal samples were also taken for comparison.

Animals were sacrificed at three weeks of age and each area of the gastrointestinal tract to be examined was tied separately to prevent the movement of the contents from one part to another. The contents of each part were plated on MacConkey's and Rogosa's agar as well as blood and neomycin blood agar. The remaining contents were removed and the mucosal surface of each of the three parts were washed gently with sterile distilled water. The washed tissue was weighed and homogenized and 1:2 dilution of the tissue homogenate plated on the previously mentioned media. This procedure was performed on all the three groups of animals and the experiment was repeated several times.

Organisms isolated from the intestinal homogenate and the contents of group III animals were further characterized to determine whether or not they were the same organism fed (MLC strain). These organisms were characterized using the API Lactobacillus

system. They were also serologically classified by the use of FA technique.

Feeding Concentrates of Streptococcus diacetilactis

Before looking at the bacterial interactions at the tissue level, it was necessary to determine the effect of feeding concentrates of a non-intestinal type of lactic acid bacteria on fecal bacterial flora. Previous work in this laboratory (Daly, 1972) indicating that S. diacetilactis was inhibitory to a variety of pathogens including E. coli. For this reason concentrates of S. diacetilactis ATCC 15346 were used in the following experiment. Frozen concentrates of the S. diacetilactis were obtained from Microlife Technics, Sarasota, Florida. The concentrate was plated on lactic agar (Elliker et al., 1956) and the organism isolated was characterized using the tests described by Sandine et al. (1962). It was also checked for acid and sodium taurocholate tolerance. The organisms were incubated at 37 C for 24 hours in lactic broth adjusted to pH 2.0; 3.0 and 4.0 using 6 N HCl. Sodium taurocholate (2%) was incorporated in lactic broth to test for bile tolerance.

A litter of nine pigs was grouped into two groups; fecal samples were taken from these pigs before putting them on test. The plating procedure was the same as described in previous experiments, except that lactic agar was used in place of Rogosa's agar to isolate S. diacetilactis. The feeding and sampling schedule was the

same as described in the Lactobacillus-feeding experiments.

Bacterial Colonization of Intestines of Pigs

To determine the type of bacteria colonizing the intestines of pigs, the following groups of animals were studied:

1. Newborn piglets collected in clean plastic bags at the time of farrowing to prevent contamination from the surroundings.
2. Conventionally-raised pigs of one week of age which were not scouring.
3. Conventionally-raised pigs of one week of age which were scouring.

The above-mentioned animals were sacrificed and the small intestines collected. The intestines were divided into nine parts and frozen sections were taken from all parts of the small intestine. Modified Gram and toluidine blue staining as described below were used. The experiment was repeated with pigs from different litters.

Staining of Tissues

The original Brown and Brenn method (1931) was slightly modified and used in these studies as follows:

1. Frozen sections were dried in an incubator at 37 C, fixed in acetone for 20 minutes and dried at 37 C for 24 hours.
2. These fixed sections were then treated with 0.05 N HCl for 3 to 4 minutes, fixed in acetone and again dried in an incubator at 37 C for 2 hours.

3. Sections were stained with freshly prepared Harris hematoxylin for 2 to 5 minutes and washed in acid alcohol until they turned light pink.

4. Sections were treated with 1% ammonia water until they turned blue and all traces of ammonia removed by washing the sections in running tap water.

5. An equal quantity of sodium carbonate and crystal violet mixed in a small tube and poured immediately onto the slide and left for 2 minutes.

6. The slide was then washed with water, dried and stained with Gram's iodine for 1 minute.

7. Sections were washed in water but not dried and then were decolorized in ether-acetone (1:1) until no blue color could be washed from the slide.

8. Sections were washed in water but not dried and stained with rosaniline hydrochloride for 5 minutes.

9. Sections were washed in water but not dried and then passed through acetone and decolorized and differentiated by dropping 1% picric acid in acetone onto the section until it turns yellowish pink.

10. Finally sections were passed through acetone, acetone-xylene (1:1) then through xylene and then mounted using a No. 1 coverslip.

Influence of Feeding Lactobacillus MLC Concentrates on Colonization Pattern

Four pigs from a litter were each collected in sterile plastic bags at farrowing. Two pigs were immediately given 20 ml of MLC concentrate and the remaining two pigs were kept as controls. They were weaned soon after birth and raised in separate boxes. The experimental pigs were fed a 1:1 mixture of Lactobacillus MLC

concentrate and sterile non-fat milk every 2 hours for the first 24 hours and every 4 hours for the subsequent 12 hours. The control pigs were given only sterile nonfat milk under the same schedule. Scouring data were recorded in both groups of pigs. Both of the experimental pigs were sacrificed at 36 hours of age; controls were found dead when checked at this time. Small intestines collected from these pigs were divided into nine parts and frozen sections taken as before from the first nine parts of the small intestine. Modified Gram and toluidine blue staining procedures as described above were used. The experiment was repeated several times with pigs from various litters.

Effect of Various Intrinsic and Extrinsic Factors on Colonization by EEC and Lactobacilli in Pig Intestine

It is well known that colostrum, normal immunoglobulins, and early weaning play an important role in the post-natal survival of piglets. In view of this, work was done to determine the influence of these factors on colonization by lactobacilli and E. coli and to measure EEC challenge effects, once colonization by exogenous lactobacilli had occurred.

There are many reports showing the inhibitory properties of human colostrum on EEC. These reports indicate that, in addition to the immunoglobulins present in colostrum, there are certain iron-binding proteins which are inhibitory to EEC in infants. These

iron-binding proteins are called lactoferrin and transferrin. Human milk has significant quantities of these but there is only one report on these iron-binding proteins in sow's colostrum, suggesting that the amount present is so small compared to human milk that they do not play an important role in the inhibition of EEC.

In our study the possible inhibitory property of the colostrum against E. coli 09:K:NM was tested; the method described by Bullen et al. (1971) was used. Colostrum was collected under non-sterile but aseptic conditions and centrifuged at 45,000 g for 45 minutes. The fat layer was removed and the supernatant distributed in 5 ml quantities in sterile tubes as follows:

Tube A	Supernatant only (5 ml)
Tube B	5 ml of supernatant plus 0.5 ml of 0.1% ferric citrate plus 0.1 ml of E. coli culture
Tube C	Supernatant plus 0.1 ml of E. coli culture

Ferric citrate was used to saturate the iron-binding capacity of the lactoferrin. All tubes were incubated at 37 C for 24 hours and plated on MacConkey's agar.

To determine whether or not normal serum would inhibit the growth of Lactobacillus MLC and EEC, an agglutination inhibition test was done using both the organisms. A standard agglutination was also done with 1:10, 1:20, 1:40, 1:80 and 1:160 antibody titers against both E. coli and Lactobacillus MLC; sterile serum (5 ml)

was added to 5 ml of MRS broth inoculated with the MLC strain and incubated at 37 C for 24 hours along with the appropriate control. The resulting cultures were then plated on Rogosa's agar.

A litter of piglets collected in clean plastic bags at the time of farrowing were weaned immediately and grouped as indicated in Table 2.

Table 2. Grouping of Animals for Colonization Studies.

Animals	Feeding and Management Conditions
Group I	No colostrum was given; animals were fed only sterile nonfat milk every 2 hours for the first 24 hours and every 4 hours for the next 48 hours.
Group II	10 ml of concentrate of MLC strain were given; following this and every 2 hours a 1:1 mixture of concentrate and sterile nonfat milk was fed for the first 24 hours and once every 4 hours for the next 48 hours.
Group III	10 ml of concentrate of MLC strain were given following this and every 2 hours, a mixture of concentrate and sow's colostrum was fed for the first 24 hours. For the next 48 hours, a 1:1 mixture of concentrate and sterile nonfat milk was fed instead of colostrum.
Group IV	10 ml of MLC concentrate were fed and the animals were left with the sow and not weaned.
Group V	10 ml of MLC concentrate were fed followed by 5 ml of serum from a lactating sow. Subsequently a mixture of 10 ml of concentrate and 10 ml of sterile nonfat milk and 5 ml of serum was fed once every 2 hours for the first 24 hours and then a 1:1 mixture of concentrate and sterile milk every 4 hours for the next 48 hours.

All the pigs were on test for 72 hours during which time some pigs died both in the control and test (group V) groups. After 72 hours, one pig each from group II, group III and group IV was sacrificed and the intestines were collected. The pigs in group I died at about 36 hours, at which time the intestines were collected; at this period, pigs from group IV and V were also sacrificed. Scouring data on all of these pigs were regularly recorded. The intestines collected were processed as described earlier. However, to obtain direct evidence of colonization by the strain of Lactobacillus-fed, fluorescent antibody technique was adopted in addition to Gram and toluidine blue staining.

Fluorescent Antibody Technique

Antigen used. A three-liter quantity of the Lactobacillus MLC was grown in MRS broth and the cells were harvested and washed in normal saline six times. The number of organisms per ml was determined by plating serial dilutions on Rogosa's agar. The suspension was mixed in a 1:1 concentration with complete Freund's adjuvent (Difco) as described in the Difco Manual Supplement (1972). This was stored at 4C, fresh antigen was prepared every month.

Production of antisera. Rabbits were given the antigen intramuscularly by the following schedule:

First week . . . . . 0.5 ml

Second week . . . . . 1.0 ml

Third week . . . . . 1.0 ml

Fourth week . . . . . 1.0 ml

Fifth week . . . . . 1.0 ml

Sixth week . . . . . 1.0 ml

Eighth week . . . . . 1.5 ml

The rabbits were bled a week after receiving the last dose of antigen. The agglutination titer of the antisera was determined using a standard saline suspension of Lactobacillus organisms. The standard tube agglutination test was used. To prevent auto-agglutination of the antigen, cells were exposed to 60 C for 30 minutes. Both the direct and indirect methods of FA staining were used.

Direct Fluorescent Antibody Technique

Antibody labelling technique: The procedure described by Formal (1972) was used with some modifications. The materials used were as follows:

1. Fluorescin isothiocyanate crystalline (Difco)
2. BSA Rhodamine counterstain (Difco)
3. 0.5 M carbonate-bicarbonate buffer (pH 9.0)
4. Phosphate buffered saline (pH 7.0)

The following procedure was adhered to:

1. Antiserum (3.0 ml) against MLC strain of Lactobacillus produced in rabbits was diluted 1:2 with cold normal saline (2 to 4 C) in the cold laboratory.

2. A 100% saturated ammonium sulfate solution was added dropwise to the serum stirred with a magnetic stirrer (9 ml of ammonium sulfate when added gave a 50% concentration in the serum).

3. After the ammonium sulfate and the serum were thoroughly mixed, the mixture was left to stand unstirred in the cold room.

4. It was then centrifuged at 9000 rpm for 5 minutes at 0 C and the pellet resuspended in 3.0 ml of 0.85% saline.

5. This suspension was then placed in a previously steamed dialysis tubing and dialysed against water for 8 hours with a change of water every 4 hours.

6. It was then dialysed overnight against 0.85% normal saline at 4 C.

7. The protein concentration was determined using modified Lowry's method (Lowry et al., 1951).

8. The volume of the serum was adjusted such that it had 10 mg/ml of total protein, i.e., if total protein was 219.75 mg then a 21.9 ml-volume contained 10 mg/ml.

9. Carbonate buffer, pH 9.0 was then added to 10% of final volume, i.e., in 21.9 ml of final volume, 2.19 ml was carbonate buffer.

10. The remaining volume was made up with normal saline and the mixture stirred well at 0 C in a closed container.

11. Fluorescin isothioscyanate was weighed at the rate of 0.02 mg/mg of protein and the dye was slowly added to the stirring serum mixture, which was then stoppered and stirred overnight.

12. A G-25 coarse sephadex column was prepared and the dye-serum mixture was placed in the column. Two layers formed in the column; a layer of densely colored dye stayed on top, whereas the conjugated globulin came down.

13. The conjugated protein was collected and filter sterilized through a millipore filter (0.45 μ) and the product was ready for use.

The following steps were adopted in staining tissues with the fluorescent antibody:

1. Frozen sections of the intestines were dried in an incubator at 37 C for 6 to 8 hours.

2. They were then fixed in acetone for 20 minutes and dried again at 37 C for 24 hours.

3. They were treated with 0.05 N HCl for 3 to 4 minutes and fixed in acetone for 20 minutes and dried at 37 C for 12 to 24 hours.

4. The sections were treated with FA rhodamine counterstain* for 30 minutes at 37 C and washed in FA buffer for 20 minutes, changing buffer once every 5 minutes.

5. Anti-Lactobacillus MLC serum (obtained from rabbit) was applied on the section and incubated at 37 C for 30 minutes.

6. Slides were washed for 20 minutes in FA buffer changing the buffer once every 5 minutes and sections were then stained with goat anti-rabbit globulin for 30 minutes at 37 C.

7. Sections were washed in FA buffer for 20 minutes with change of buffer once every 5 minutes.

8. Sections were dried and mounted using a No. 1 coverslip and FA mounting fluid and examined under the UV microscope.

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* Normal bovine serum conjugated with lissamine rhodamine tissue fluoresces red and organisms do not take up the stain.

Each of these pigs was given 2.5 ml of E. coli 09:K:NM cell suspension; the feeding schedule was the same as described in the earlier experiment. All pigs except the virulence control animals in group I received Lactobacillus concentrate and sterile milk. The control pig was left with the sow for the first 24 hours to provide colostrum, it was then taken from the sow and raised in another box for 18 hours on sterile nonfat milk only. At this age it was given 2.5 ml of E. coli suspension.

All the above pigs were on the test for 72 hours; scouring results were recorded. At 72 hours after feeding E. coli, pigs were sacrificed and intestines were processed in the same manner as described before.

Effect of Postnatal Feeding of MLC Concentrates on Fecal Coliforms and Scouring

From earlier experiments it was observed that feeding MLC concentrate from birth had much more influence on intestinal coliforms and scouring than feeding at later stages of life. Therefore, experiments were conducted to examine the feasibility of early feeding as a herd management practice to reduce the incidence of scouring.

Eight litters of pigs were used in this experiment; four litters were controls and four were test litters. All the pigs were left with the sow and were not weaned until about five weeks of age.

Immediately after birth, all the experimental pigs were fed about 10 ml of MLC concentrate. Following this, they were given another 10 ml of concentrate at 12 and 36 hours of age and following this, once every third day until weaning. Samples were plated only on MacConkey's and Rogosa's agar.

In Vitro Inhibition Studies

Inhibitory Studies in Milk and Lactic Broth

The following organisms were used as indicator organisms: E. coli (β-hemolytic) and S. aureus, both obtained from pig intestine, and a virulent strain of Salmonella typhimurium obtained from the Veterinary Diagnostic Laboratory at Oregon State University. Each indicator organism was grown in association with the MLC strain of Lactobacillus in lactic broth and viable counts were obtained by plating on the approximate medium. One hundred ml of lactic broth in a 250 ml Erlenmeyer flask were inoculated (one percent) with an actively growing culture of strain MLC and the indicator organism. Control flasks had each organism growing alone; incubation was static at 37 C and viable counts were determined at intervals. MacConkey's agar was used to count E. coli and S. typhimurium and Vogle-Johnson agar was used to count S. aureus.

Estimation of Acids Produced by Lactobacillus MLC

The concentrate strain of Lactobacillus was found to have all the characteristics of a homofermentative bacterium. To further ascertain this, it was examined for its ability to produce different acids. The acid production was studied in milk and lactic broth cultures without added sodium acetate. Total lactic acid was determined by the Harper and Randolph (1960) modification of Ling (1951) method as outlined by Mattsson (1965). The amount of lactic acid in cell-free supernatants was determined colorometrically from the yellow color of the supernatant on addition of ferric chloride. A 2% $FeCl_3 \cdot 6H_2O$ solution in water was used. The optical density at 425 nm was read within 15 minutes using a Gilford (Model 2000) automatic sprectrophotometer. An uninoculated blank was treated similarly. The type of lactic acid produced was determined using the method described by Mattsson (1965); the nicotinamide adenine dinucleotide (NAD grade III) and lactic dehydrogenase were obtained from Sigma Chemical Company. The optical density was read at 340 nm (Bausch and Lomb spectronic 20) against an uninoculated blank treated in the same way as the sample. The amount of L(+) lactic acid was read from a standard curve.

Culture supernatants were checked for volatile acids by gas liquid chromatography. A 6' X 1/8" stainless steel column was filled by vibration with a packing mixture prepared as follows:

2.0 gm of neo-pentyl glycol succinate and 0.2 gm of concentrated phosphoric acid were dissolved in about 50 ml of chloroform (enough to form a smooth slurry with poropak Q). Once dissolved, 10 gm of 80 to 100 mesh poropak Q were added, and the slurry well-stirred in a beaker. The mixture was evaporated to dryness and added to the column by vibration. The column was equilibrated for 21 hours at 180 C before use. All analyses were made using an F and M high efficiency gas chromatograph (Model 402) with a Honeywell Strip Chart Recorder (Electronik 16) and a Hewlett-Packard 3370 A integrator. Isothermal operation at 160 C was used. Injection port and detector temperatures were 198 C and 195 C respectively. Gas flow rates were approximately as follows: Helium 70 ml/min, hydrogen 20 ml/min and air 300 ml/min. The volume of the sample used was 1 to 2 μl.

Inhibitor Produced by *Lactobacillus* MLC

Isolation and Identification of the Inhibitor

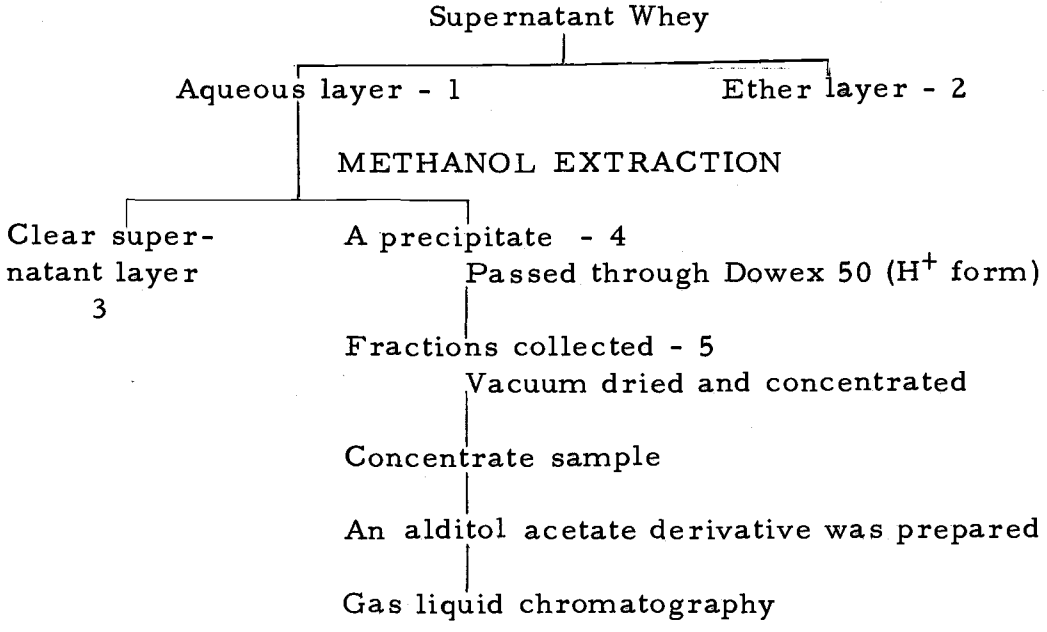
The *Lactobacillus* concentrate obtained from Microlife Technics, Sarasota, Florida, was used throughout this phase of the study. *S. aureus* type C was the main indicator strain; in addition, strains of *E. coli* and *S. typhimurium* were used.

The organism were grown in sterile nonfat milk. A ten percent inoculum was used. One hundred ml of canned concentrate were

added to one liter of sterile nonfat milk, it was incubated at 37 C for 72 hours under aerobic conditions. The coagulated milk was then centrifuged at 5000 rpm for 40 minutes and the supernatant whey was used for further work.

The pH of this supernatant was determined and an aliquot (3.0 ml) was added to 10 ml of lactic broth which was then inoculated with one percent S. aureus and incubate at 37 C; as a control, 3.0 ml of phosphate buffer, pH 4.0, was added to 10 ml of lactic broth which also was inoculated with S. aureus.

The steps followed in purifying and identifying the inhibitor produced by Lactobacillus MLC is described below:



Fractions one through six were checked for sugars by the anthrone and proteins by the ninhydrin tests. Tests showed that

fraction 5 had most of the inhibitor. For further purification, fraction 5 was passed through a Dowex-50X hydrogen form column. Eight fractions of 25 ml each (first eight) were collected. Fraction 2 through fraction 6 gave a positive anthrone reaction and were combined and concentrated by vacuum drying to about 6 ml. This concentrated solution was used for further work.

Paper chromatography was used both to fractionate and concentrate the inhibitor. The chromatogram was developed in water: proponal solvent system (1:9) only in one direction and sprayed with p-anisidine. Attempts to concentrate the inhibitor by paper chromatography were not successful.

It was decided to use gas liquid chromatography (GLC) for further identification of the component. An alditol acetate derivative of the concentrate was prepared as follows:

1. Five ml of the concentrated (fraction 5) sample were taken and the pH adjusted to 5.5 using saturated barium hydroxide.

2. Sodium borohydride (300 mg, Ventron Corporation) powder and 1 ml of myo-inositol standard (10 mg/ml) were added and the reaction allowed to proceed for 30 minutes.

3. The foam formed during the reaction was removed by adding glacial acetic acid and the sample was evaporated in a vacuum drier.

4. This dried sample was then extracted with 10 ml of methanol and vacuum dried. This methanol extraction was repeated three times.

5. After the third extraction, it was evaporated thoroughly and all the moisture was removed by drying in an oven at 110 C for 10 minutes.

6. Sodium acetate anhydrous (500 mg) and 10 ml of acetic anhydride were added and refluxed for 20 minutes.

7. The preparation was then cooled to room temperature and evaporated in a vacuum drier.

8. The material was then dissolved in 15 ml of dichloromethane and centrifuged. The clear supernatant was collected and evaporated to one ml by bubbling with nitrogen gas.

9. The concentrated material was analyzed by GLC.

Preparation of Column

One hundred mg of ethylene glycol succinate (F and M Scientific Corporation) were dissolved in 25 ml of chloroform and 100 mg of ethylene glycol adipak (Applied Sciences Laboratory, State College, Pennsylvania) were dissolved in acetone. The above mixtures were quickly poured together, mixed with 10 gm of Gas Chrom P (100 - 200 mesh, Applied Science Laboratory), and occasionally stirred gently. After 30 minutes the mixture was allowed to drain and the residue dried at room temperature in a large Petridish. About 6 ml of this powder was packed, with vibration into a 4' X 1/8" o.d. stainless steel column. Both ends of column were plugged with glass wool and conditioned overnight at 180 C by purging with carrier gas (helium).

Carrier gas (helium), 60 lb/sq in.; Hydrogen, 3 mm; Air, 3 mm; Oven temperature, 186 C; Flash heater, 205 C.

Sample (2.0 µl) was injected into the column. Before running the test sample, a standard preparation of sugars was run through the column to obtain retention times for each sugar.

Inhibitory Studies Using 2-Deoxy-D-glucose

Pure 2-deoxy-D-glucose was obtained from Sigma Chemical Company and its inhibitory properties studied in a basal medium containing Tryptone, 20 gm; gelatin, 2.5 gm; sodium acetate, 1.5 gm; sodium chloride, 4.0 gm. Because glucose and galactose were also present in the sample along with 2-deoxy-D-glucose all three sugars were used in different combinations and concentrations at pH 5.0 and 7.0 as shown below.

Flask No.	Sugars Added (0.1%)
1	2-deoxy-D-glucose
2	Glucose
3	Galactose
4	Glucose and 2-deoxy-D-glucose
5	Galactose and 2-deoxy-D-glucose
6	Galactose, glucose and 2-deoxy-D-glucose
7	Glucose and galactose
8	None (basal medium control)

A one percent inoculum of the culture was made and incubation at 37 C, optical density readings were recorded at 0, 4, 8, 16, 20, 24, 36 and 42 hour intervals. Test organisms included were S. aureus which produced toxin types A, B, C, D and E as well as E. coli, Pseudomonas sp. and S. typhimurium.

Role of Antigen in Intestinal Colonization by Lactobacilli

Antigen Studies

Since colonization in the intestines by EEC is controlled by a capsular (K88) antigen, colonization by lactobacilli might also be associated with an antigen. Therefore, a study was done to determine the possible presence of soluble antigens associated with colonization by lactobacilli.

Organisms used in this study were obtained from the stock culture collection maintained in the Department of Microbiology at Oregon State University. A soluble antigen of the following organisms was made as described earlier.

Lactobacillus strain MLC

Lactobacillus strain FH

Lactobacillus salivarius ATCC 11742

Antiserum was produced only against the MLC strain in rabbits. An initial antigenic comparison was made among these three strains

of lactobacilli using the Ouchterlony technique described above. Based on these results, the above three strains were selected to give a wide range in antigenic picture. For example, the FH strain was originally isolated from pig intestine; strain MLC was known to be a colonizing type from the previous findings of the present study; L. salivarius did not show any antigenic relationship with either MLC or FH strains, so it was assumed to be a non-colonizing type.

After determining the antigenic relationship among these three strains, concentrates of these organisms were made and feeding experiments were conducted.

Feeding Experiments

Pigs were collected at the time of farrowing in clean plastic bags, weaned immediately and grouped in separate boxes as shown in Table 3.

All the pigs were on test for about 48 hours at which time they were sacrificed and the intestines collected and processed as described earlier.

Table 3. Grouping of Animals for Feeding Different Lactobacilli.

Group	Feeding Conditions
1	Fed sterile nonfat milk once every 2 hours for first 24 hours and once every 4 hours for next 24 hours.
2	Fed a 1:1 mixture MLC concentrate and sterile nonfat milk under the same schedule as in group 1.
3	Fed a 1:1 mixture of FHS concentrate and sterile nonfat milk under the same schedule as in group 1.
4	Fed a 1:1 mixture of concentrate of L. salivarius and sterile nonfat milk under the same schedule as in group 1.

RESULTS

Characterization of the Lactobacillus Organism Used in the Study

Characteristics of the Lactobacillus organism isolated from the concentrate appear in Table 4. From the results it can be seen that the organism possesses all the typical characteristics of the genus Lactobacillus. It should be noted that this organism is sensitive to sodium taurocholate in spite of its original habitat being the human intestine. Fermentation results from the A.P.I. test are shown in Table 4. From this it can be said that the organism belongs to the group Thermobacterium, since it does not ferment ribose or arabinose and does not produce gas from glucose; also, it was found to be homofermentative. Based on the various fermentation characteristics the organism was tentatively classified as Lactobacillus lactis.

An antigenic comparison of Lactobacillus MLC was made with other lactobacilli using an Öuchterlony immunodiffusion technique. A complete homology in the antigenic make up was seen between Lactobacillus MLC and Lactobacillus lactis ATCC 12315 (Figure 2).

Thermal melting of DNA was then carried out and Figure 3 shows the denaturation pattern. From the profile it may be seen that the organism had a high T_m (88.5 C). The moles percent GC content of DNA was determined using Mandel's equation (Mandel et al., 1970),

Table 4. Characterization of Lactobacillus organisms used in concentrated form in feeding experiments.

Observation	Result	Observation	Result
Origin	Human intestine[a]	Methyl-d-mannoside	+
Optimum growth conditions	Anaerobic environment at 37 C[b]	Methyl-d-glucoside	-
Growth with:		N acetyl-glucosamine	+
2% NaCl	3×10^2 cfu/ml; control: 8×10^4 cfu/ml	Amygdalin	-
		Arbutine iron citrate	-
2% sodium taurocholate	no growth	Aesculine iron citrate	+
2% sodium desoxycholate	no growth	Salicin	-
Catalase	absent	d (+) cellobiose	-
Pseudocatalase	absent	Maltose	+
Type lactic acid produced	DL	Lactose	+
Fermentation[c]:		d (+) melibiose	-
Bromcresol	-	Saccharose (sucrose)	+
Glycerol	-	d (+) trehalose	+
Erythritol	-	Inuline	-
d (-) arabinose	-	d (+) melezitose	-
1 (+) arabinose	-	d (+) raffinose	+
Ribose	-[d]	Dextrine	-
d (+) xylose	-	Amylose	-
1 (-) xylose	-	Starch	-
Adonitol	-	Glycogen	-
Methyl-xyloside	+	Arginin	-[d]
Galactose±	-	Glucose	+, no gas[d]
d (+) glucose	+	Teepol 0.4%	+
d (-) levulose fructose	+	Teepol 0.6%	-
d (+) mannose	+	NaCl 4%	+
1 (-) sorbose	-	NaCl 6%	+
Rhamnose	+	NaCl 10%	-
Dulcitol	-	ONPG	-
Meso-inositol	-	Potassium nitrate + glucose	-
Mannitol	-	Pyruvic acid (V.P.)	+
Sorbitol	-		

[a]Isolated by S. Farr, Microlife Technics, Sarasota, Florida

[b]95% nitrogen, 5% CO_2 atmosphere in evacuated metal cylinder

[c]API test pack system

[d]These characters, when negative, are typical of Thermobacteria

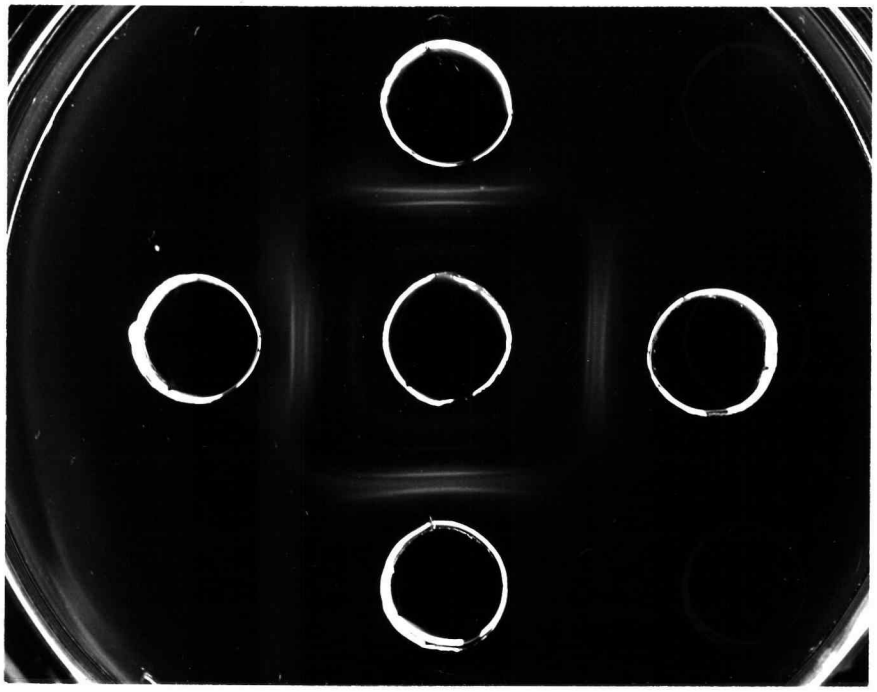


Figure 2. An Öuchterlony immunodiffusion test to show homology between Lactobacillus MLC and L. lactis ATCC 12315. The center well contained MLC antiserum with homologus antigen in the left and right wells; the top and bottom wells contained L. lactis antigen.

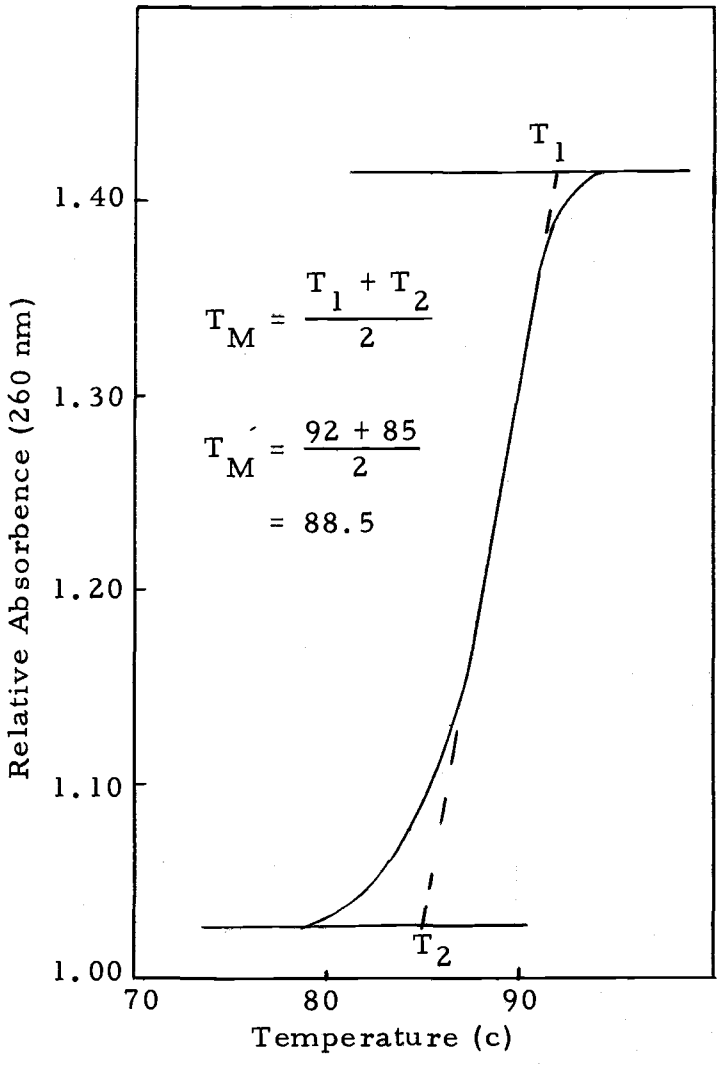


Figure 3. Absorbance - temperature denaturation profile of Lactobacillus MLC DNA.

and it was found that the organism had a high GC content of 48 moles % GC.

DNA-DNA hybridization experiments using ^{3}H labelled DNA showed 87% homology with L. lactis 12315 (Table 5). Thus Lactobacillus MLC used in the feeding experiments was identified as Lactobacillus lactis. Its lack of similarity with L. acidophilus also was emphasized since the latter bacterium has a low GC content (36 moes % GC).

Table 5. Hybridization Data Obtained When Tritium Labelled DNA from Lactobacillus lactis ATCC 12315 was allowed to reassociate with homologous and heterologous DNA samples.

DNA Source	Cpm	Depression	% Homology
L. lactis ATCC 12315 (^{3}H label)	2228	-	-
L. lactis ATCC 12315	160	2068	100
Lactobacillus MLC	429	1799	87
L. salivarius ATCC 11742	2364	0	0
L. bulgaricus ATCC 11842	733	1495	72

Normal Fecal Bacterial Flora

Table 6 shows the numbers of different genera of bacteria recovered from fecal samples taken at random from pigs of different ages in the Oregon State University Swine herd. The data generally confirm the findings of Smith and Crabb (1961) on the numbers and types of bacteria found in pig feces. From the Table 7 it may be seen that 32×10^9 cfu/gm feces for the total (arithmetic sum) anaerobic count on selective media compares favorably with 67×10^9/gm for the total anaerobic count found on brain heart infusion blood agar. However, the aerobic counts on selective media (12×10^8 cfu/gm) were about one log lower than the total aerobic count on blood agar. This indicated that the selective media were inhibiting some coliforms and or staphylococci, or that other bacteria such as Gram-negative cocci were numerically important in the aerobically incubated samples.

As described in the review of literature, the balance among intestinal bacteria is important in maintaining normal health. For unknown reasons stress conditions cause changes in the balance. It is well known that swine are prone to scour at three stages in their life, the first and third weeks of age and immediately after weaning. It was of interest in this study to determine how the balance between desirable (lactobacilli) and undesirable (EEC or coliform) bacteria

Table 6. Normal Fecal Bacterial Flora in Pigs at Various Ages.

Age	cfu/gm of Feces					
	Coliforms	Lactobacilli	Streptococci	Staphylococci	Bacteroides	Clostridia
1st week	500,000,000	800,000,000	400,000,000	20,000	400,000,000	200,000
2nd week	1,000,000,000	1,000,000,000	60,000,000	5,000,000	1,600,000,000	3,000,000
Weaned (6 weeks)	600,000,000	3,000,000,000	90,000,000	100,000	740,000,000	3,000,000
Adult	300,000,000	2,000,000,000	2,000,000	100,000	140,000,000	700,000

Table 7. Bacterial Flora of Pig Feces Determined on Selective Media.

Organism	Cfu/gm	
	Selective Media	Blood Agar
Coliforms	1,000,000,000	
Staphylococcus aureus	220,000,000	
Sum of the aerobic counts[a]	1,200,000,000	
Total aerobic counts		59,000,000,000
Bacteroides sp.	1,100,000,000	
Clostridium sp.	14,000,000	
Lactobacillus sp.	26,000,000,000	
Streptococcus sp.	5,000,000,000	
Sum of anaerobic counts	32,000,000,000	
Total anaerobic counts		67,000,000,000

[a] Arithmetic sum to two significant figures.

changed at the time of scouring. The results are presented in Table 8 and Figure 4. From the results it can be seen that at the time of scouring the numbers of coliform bacteria suddenly increased but the lactobacilli did not. This pattern of increase in coliforms was seen in the majority of the scouring pigs examined. In the case of healthy non-scouring pigs, the Lactobacillus counts were invariably higher than coliform counts (Figure 4 and Table 8).

Feeding Experiments

Before each feeding experiment and at least once each week, serial dilutions of each lot of thawed concentrate were plated on Rogosa's medium. Concentrate was stored for a maximum of 30 days and never decreased significantly in total counts which ranged from 50 to 90 X 10^9 cfu/ml.

Bottle Feeding

Results of the first experiment are shown in Figures 5 and 6. It can be seen that there was not much reduction in coliform counts up to the weaning period (8 weeks); also at the time of weaning the ratio of Lactobacillus to coliforms was almost 1:1. However, when an increased amount (30 ml) of the concentrate was fed, the next sampling (two days later) showed a sudden decrease in the coliform counts (Table 9). This indicated that the quantity of concentrate given

Table 8. Changes in the Fecal Coliform and Lactobacilli Counts at the Time of Scouring.

Fecal Consistency	Cfu/gm[a]	
	Coliforms	Lactobacilli
Normal	200,000,000	20,000,000,000
Normal	400,000,000	20,000,000,000
Normal	80,000,000	20,000,000,000
Normal	200,000,000	20,000,000,000
Scouring	19,000,000,000	20,000,000,000
Scouring	22,000,000,000	18,000,000,000
Soft	14,000,000,000	20,000,000,000
Soft	6,000,000,000	15,000,000,000

[a] Data represent bacterial counts from fecal samples taken at three-day intervals.

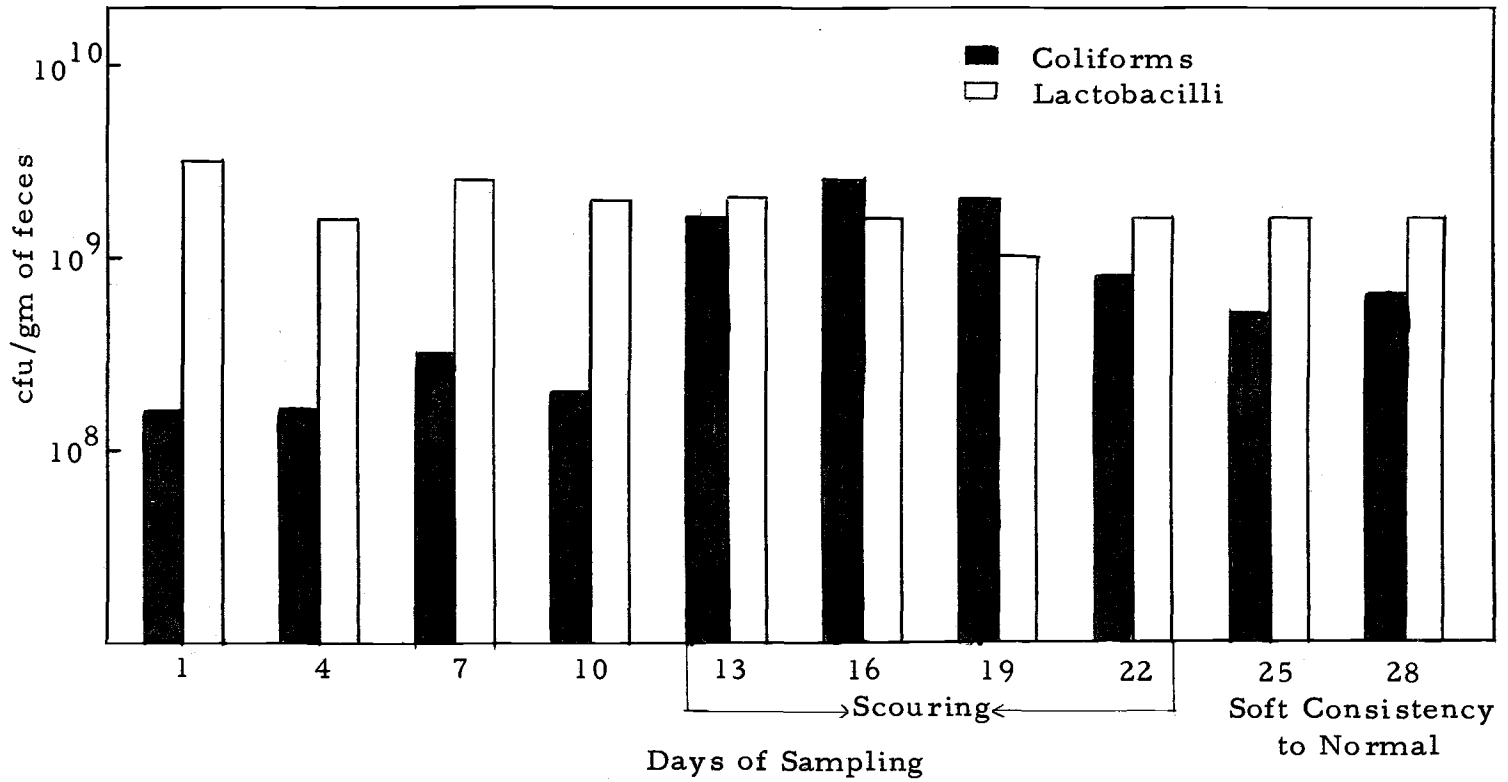


Figure 4. Histogram Showing the Fecal Coliform and Lactobacilli Counts from Birth to Four Weeks of Age.

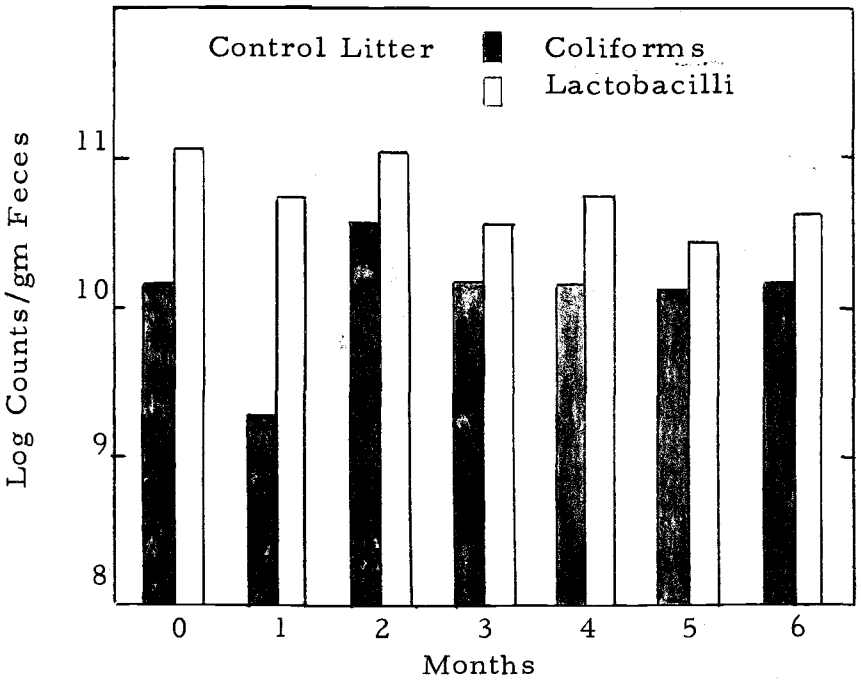


Figure 5. Histogram Showing the Fecal Coliform and Lactobacillus Counts in Control Pigs.

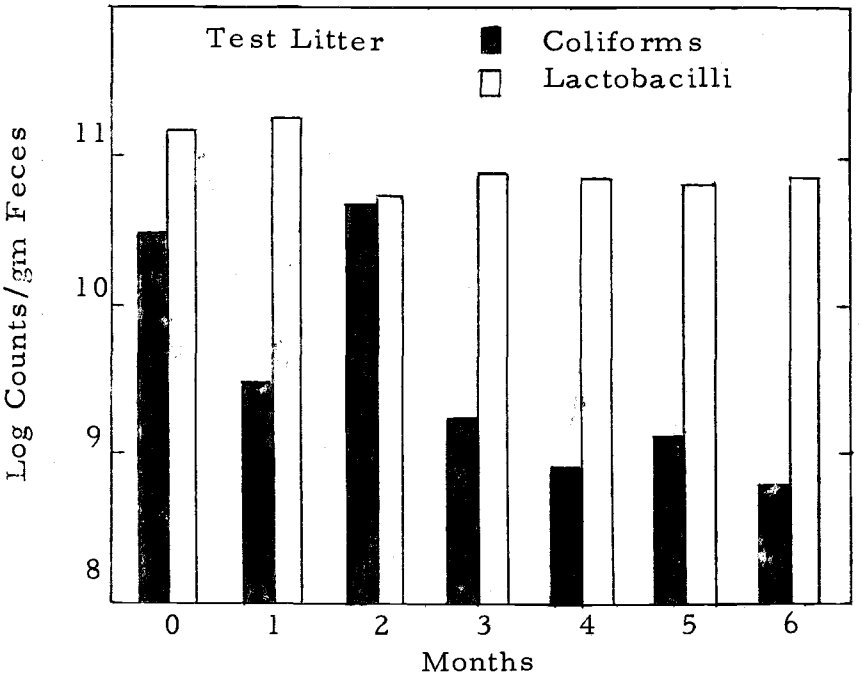


Figure 6. Histogram Showing the Fecal Coliform and Lactobacillus Counts in Pigs Fed Concentrates of Lactobacillus MLC.

Table 9. Data Showing a Decrease in Coliform Counts Following an Increase in the Dose of Lactobacillus MLC Concentrate Fed From 15 to 30 ml per Pig per Day.

	Coliform Counts[a]	Lactobacillis Counts[a]
Before increasing the dosage of MLC concentrate	600,000,000[b]	20,000,000,000
	1,800,000,000	15,000,000,000
	1,100,000,000	10,000,000,000
Two days after increasing the dosage of MLC concentrate to 30 ml	10,000,000	20,000,000,000
	11,000,000	20,000,000,000
	10,000,000	20,000,000,000

[a] Counts represent average of four pigs.

[b] Counts obtained from fecal samples taken from test pigs once every two days.

early in the experiment was insufficient. At the end of six months, a 95% decrease in the coliform counts was found in comparison to the control animals but little reduction in the severity of scouring was noted.

Results of the next feeding with a different schedule appear in Figures 7 and 8. Here a gradual decrease (Fig. 8) in the coliform counts can be seen; also, there were no rise in coliform counts either at the second or third week of age nor after weaning. After 90 days of Lactobacillus feeding there was almost a 99.9% decrease in coliform counts. The E. coli counts of control pigs (Fig. 7) were about 700,000,000 per gram (average data of four pigs) while the counts from pigs fed concentrates of MLC Lactobacillus for 54 days were only 600,000 per gram (average of five pigs). Also, the incidence, duration and severity of scouring was very low in test animals. Following removal of the pigs from the Lactobacillus diet, the continuing effect of the organisms in suppressing coliform organisms was evident. This may be seen in Figures 9 and 10. Even 30 days after the last feeding (Fig. 10) the coliform counts were still at the same level as observed at the time of feeding.

Feeding Through the Drinking Water System

Following these studies, attempts were made to determine the feasibility of feeding lactobacilli to a large number of pigs in a

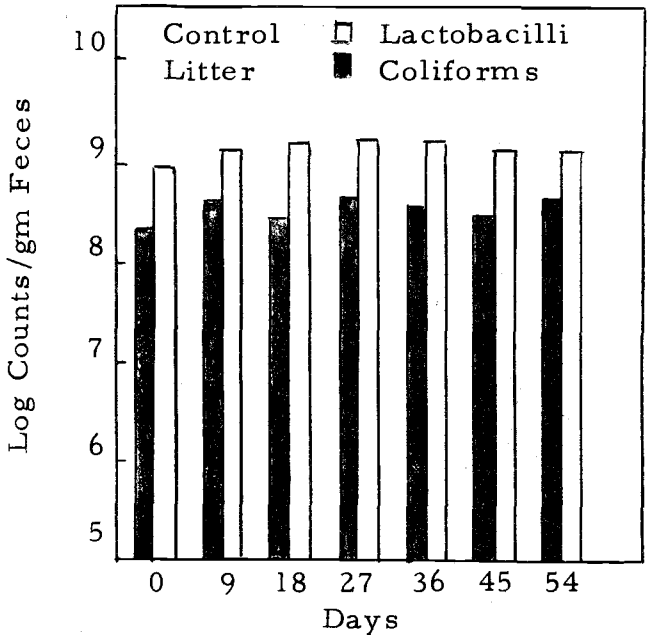


Figure 7. Histogram Showing the Fecal Coliform and Lactobacillus Counts from Pigs from the Control Group.

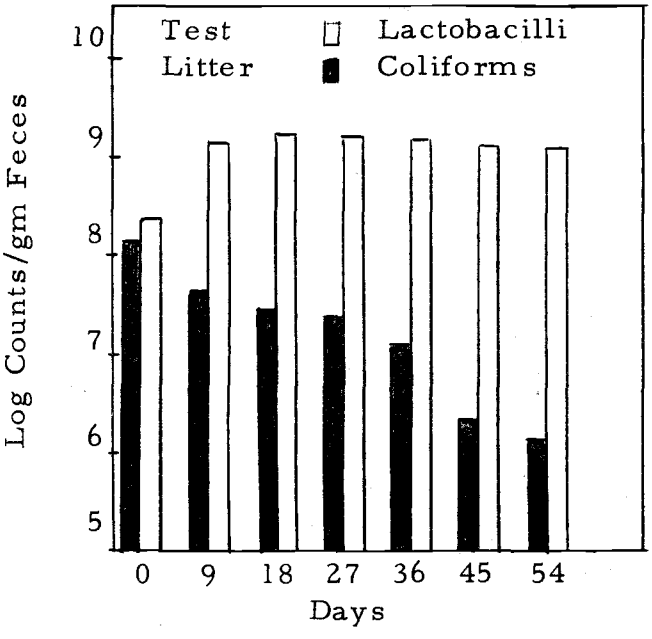


Figure 8. Histogram Showing the Fecal Coliform and Lactobacillus Counts in Pigs Fed Concentrates of MLC Strain.

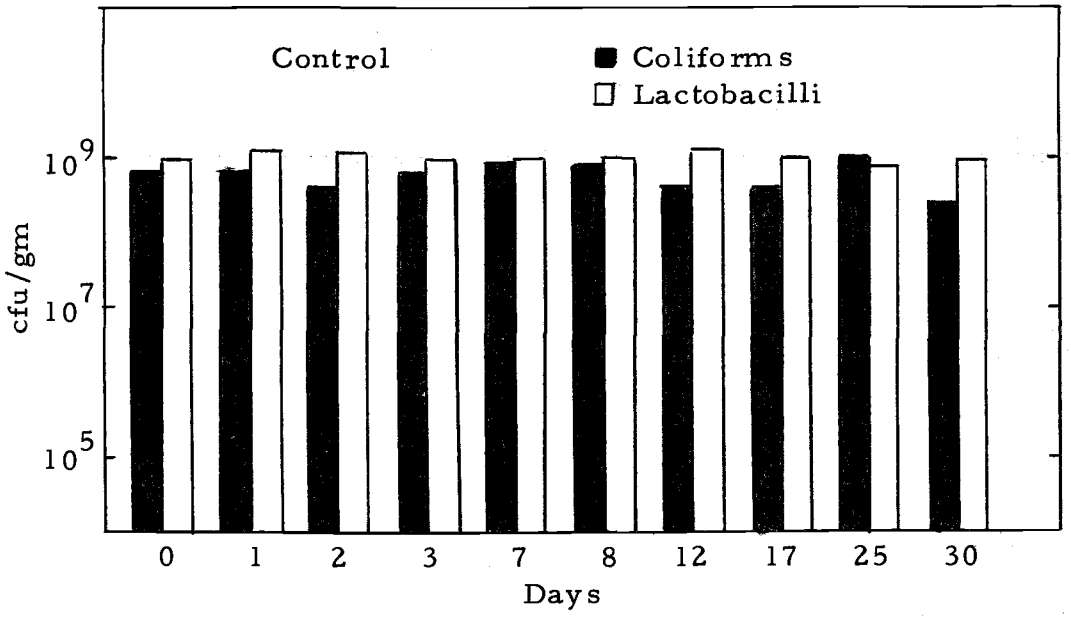


Figure 9. Histogram Showing the Fecal Coliform and Lactobacillus Counts from Control Pigs.

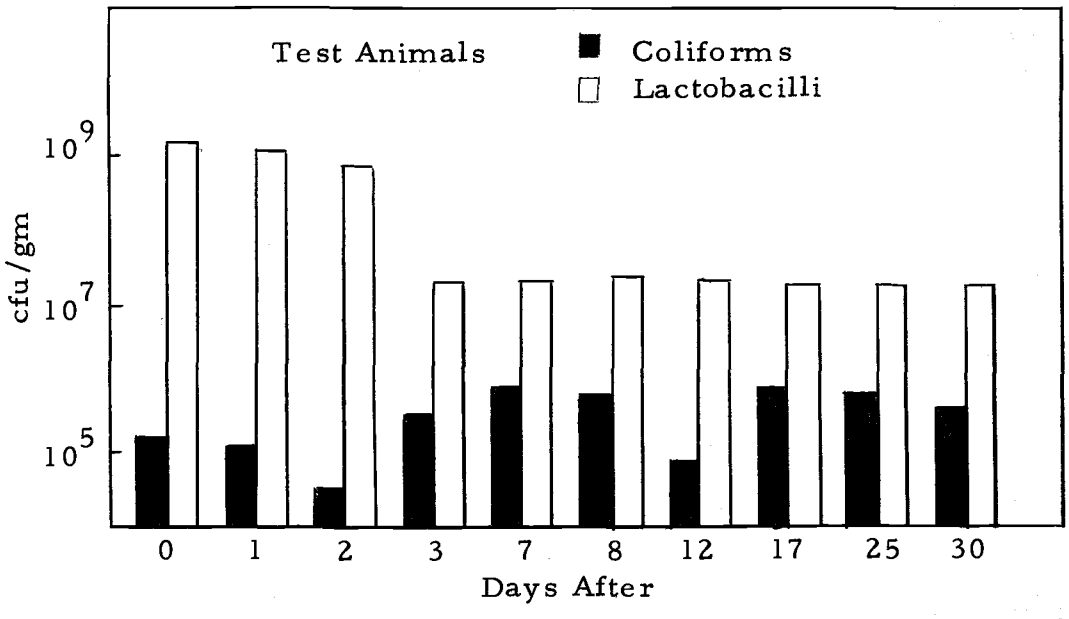


Figure 10. Histogram Showing the Fecal Coliform and Lactobacillus Counts from Pigs Discontinued Feeding Concentrates of Lactobacillus MLC. Samples taken from the day of stopping feeding for 30 days.

herd. Since this was to be attempted through the drinking water system, survival of Lactobacillus MLC in water with or without lactose was studied. Results (Table 10) indicated that the organism survived for at least 36 hours without significant loss in viability. The acid-producing ability of the organism was not studied. Daily plating of the concentrate and drinking water mixed with concentrate showed approximately the same number of organisms on successive days in each case. The final concentration of lactose in the drinking water was approximated at one percent.

The changes in the fecal coliform and lactobacilli are shown in Tables 11 and 12. Results are expressed as average of plate count data from fecal samples grouped by the number of days the animals were on test. There was a 90% reduction in the coliform counts after 90 days of feeding concentrates of Lactobacillus MLC (Table 12). In addition there was no increase in coliform counts either at 2 to 3 weeks of age or after weaning. As in earlier experiments, there was a negligible increase in lactobacilli counts. The reason for this is not known but because of the reduction in coliform counts, the Lactobacillus to coliform ratio was maintained at a high level in all the experimental animals. This ratio after 40 days of feeding reached as high as 166.11:1 in the Lactobacillus-fed animals; whereas, in control animals at the same period, the ratio was 2.16:1.0, indicating a definite change in the microbial balance.

Table 10. Viable Counts (cfu/ml) of MLC Lactobacilli Found with Time in Frozen and Diluted Concentrate in the Water Distribution System.

Concentrate		Water	
Hours	Cfu/ml x 10^{10}	Hours	Cfu/ml x 10^{8}
1/2	8.1	1/2	3.8
3	2.8	1/2	1.8
4	1.9	1	1.7
14	5.9	3	0.4
18	4.5	4	0.9
24	4.7	12	4.6
36	6.4	14	3.0
48	7.2	18	1.2
>72	<0.3	24	3.7
		36	1.9

Table 11. Fecal Lactobacillus and Coliform Means[a] and L/C Ratios of Control Pigs from 12 Pens[b].

Days Age	Lactobacillus	Coliforms	L/C Ratio
14-20	3.09	3.86	0.80
21-27	3.10	3.60	0.86
28-34	2.87	2.81	1.02
35-41	4.72	2.19	2.16
42-48	15.90	1.23	12.93
49-55[c]	11.10	2.88	3.85
56-62	6.42	0.28	23.18
63-69	9.30	0.27	34.44
70-76	10.80	1.14	9.47
77-83	7.86	2.00	3.93
84-104	12.88	3.33	3.87

[a] Geometric mean of Cfu/gm of Feces x 10^8.
[b] Three pigs sampled at random from each pen weekly.
[c] First samples after weaning.

Table 12. Fecal *Lactobacillus* and Coliform Means[a] and L/C Ratios of Lactobacillus-Treated Pigs from 12 Pens.[b]

Days from Start[c]	*Lactobacillus*	Coliforms	L/C Ratio
0- 5	2.94	2.90	1.01
6-12	4.78	2.02	2.37
13-19	5.38	0.40	13.42
20-26	4.63	0.49	9.39
27-33	6.41	0.34	19.13
34-40	8.97	0.05	166.11
41-47	8.95	0.13	70.47
48-54[d]	18.50	0.29	64.01
55-61	13.40	0.49	27.13
62-68	13.80	0.36	38.76
69-75	12.80	0.31	41.29
76-82	12.40	0.39	31.71
83-91	7.24	0.20	36.94

[a] Geometric mean of Cfu/gm of Feces x 10^8.
[b] Three pigs sampled at random from each pen weekly.
[c] Start at 2 weeks of age.
[d] First samples after weaning.

Effects of Feeding MLC Concentrate on the Bacterial Flora of Intestinal Parts

After studying the gross changes in the fecal bacterial flora, changes in the flora of different parts of the gastrointestinal tract were examined. The results are given in Tables 13, 14, 15 and 17. All the colonies on MacConkey's agar from the scouring animal (Table 13) were found to be E. coli; the scouring pig had 10^9 E. coli/ gm of the feces. Furthermore, from Table 14 it can be seen that all the hemolytic E. coli in the intestines of the scouring pig were found in the tissue homogenate and none in the contents of the intestine. In contrast to this, no hemolytic E. coli were found in the experimental pigs. Even the non-hemolytic E. coli were found (Table 14) in numbers 2 to 3 log lower in the homogenate of the experimental pig. Regarding the changes in the lactobacilli (Table 15), intestinal homogenate of the scouring animals and lower numbers than the experimental pig. Also, the homogenate of the upper part of the intestines had almost 95% higher numbers of lactobacilli than were found in the scouring pig. This suggested that the lactobacilli were becoming established in the area, thereby reducing the colonization by E. coli and reducing the chances of scouring. The lactobacilli isolated from the tissue homogenate of these pigs were characterized using the API biochemical system as well as serologically by FA staining. The results are presented in Table 16 and Figure 11.

Table 13. Numbers of E. coli Found in Contents (C) and Homogenate (H) of Different Parts of the Small Intestine.

Animal	Proximal		Distal		Feces
	C	H	C	H	
Control	1,500,000	140,000	7,000,000	220,000	780,000,000
Scouring	400,000	600,000	2,700,000	30,000	10,000,000,000
Lactobacillus Fed	30,000	300	20,000	1,000	10,000

Table 14. Total Aerobic Bacterial Counts Found in Contents (C) and Homogenate (H) of Different Parts of the Small Intestine.

Animal	Blood Reaction	Proximal		Distal		Feces
		C	H	C	H	
Control	Hemolytic	140,000	100,000	2,000,000	7,000	
	Non-hemolytic	10,000	100,000	10,000	30,000	
Scouring	Hemolytic	10,000	150,000	10,000	4,000	9,600,000,000
	Non-hemolytic	300,000	10,000	1,600,000	1,000	10,000
Lactobacillus Fed	Hemolytic	< 1,000	< 1,000	< 1,000	< 1,000	< 1,000
	Non-hemolytic	1,600,000	8,000	580,000	40,000	2,800,000

[a] No counts were seen at 10^2 dilutions.

Table 15. Number of Lactobacilli Found in Contents (C) and Homogenate (H) of Different Parts of the Small Intestine

Animal	Proximal		Distal		Feces
	C	H	C	H	
Control	10,000,000	1,600,000	45,000,000	220,000	
Scouring	180,000,000	1,500,000	720,000,000	50,000	230,000,000
Lactobacillus Fed	940,000,000	9,300,000	620,000,000	2,700,000	970,000,000

Table 16. Characterization of Lactobacillus organisms isolated from intestinal homogenate of Lactobacillus MLC-fed pigs.

Observation	Result	Observation	Result
Origin	Intestinal homogenate of pigs fed Lactobacillus MLC concentrate	Sorbitol	-
		Methyl-d-mannoside	+
Optimum growth conditions	Anaerobic environment at 37C[a]	Methyl-d-glucoside	+
Growth with:		N acetyl-glucosamine	+
2% NaCl	-	Amygdalin	+
2 % sodium taurocholate	-	Arbutine iron citrate	-
2% sodium desoxycholate	-	Aesculine iron citrate	+
Catalase	absent	Salicin	+
Pseudocatalase	absent	d (+) cellobiose	-
Type lactic acid produced	DL	Maltose	+
Fermentation:[b]		Lactose	+
Bromcresol purple	-	d (+) melibiose	-
Glycerol	+	Saccharose (sucrose)	+
Erythritol	-	d (+) trehalose	+
d (-) arabinose	-	Inuline	-
1 (+) arabinose	-	d (+) melezitose	-
Ribose	-[c]	d (+) raffinose	+
d (+) xylose	-	Dextrine	-
1 (-) xylose	-	Amylose	-
Adonitol	-	Starch	-
Methyl-xyloside	-	Glycogen	-
Galactose	-	Arginine	-[c]
d (+) glucose	+	Glucose	+, no gas[c]
d (-) levulose fructose	+	Teepol 0.4%	+
d (+) mannose	+	Teepol 0.6%	+
1 (-) sorbose	-	NaCl 4%	+
Rhamnose	-	NaCl 6%	+
Dulcitol	-	NaCl 10%	-
Meso-inositol	-	ONPG	+
Mannitol	-	Potassium nitrate + glucose	-
		Pyruvic acid (V.P.)	+

[a] 95% nitrogen, 5% CO_2 atmosphere in evacuated metal cylinder.

[b] API test pack system

[c] These characters, when negative, are typical of Thermobacteria

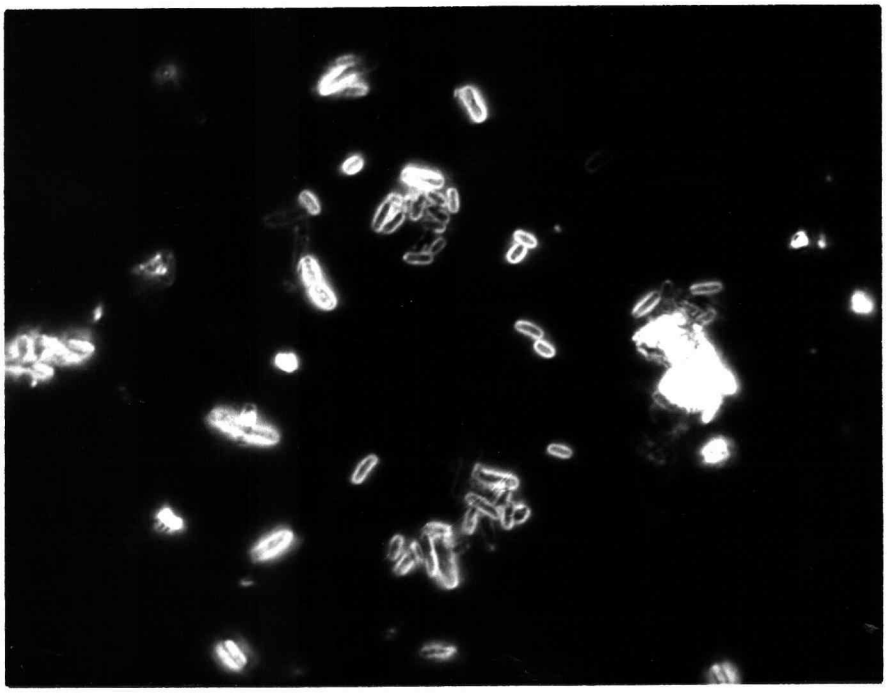


Figure 11. Fluorescent Antibody Stained Lactobacillus Strain Isolated from Tissue Homogenate of Lactobacillus MLC Fed Pigs.

Table 17. Bacteroides Counts Found in Content (C) and Homogenate (H) of Different Parts of the Small Intestine.

Animal	Proximal		Distal		Feces
	C	H	C	H	
Control	13,000,000	700	200,000	300,000	-
Scouring	7,000,000	2,600,000	30,000,000	300,000	92,000,000
Lactobacillus Fed	940,000,000	60,000	620,000,000	10,000	190,000,000

From the results it can be seen that this Lactobacillus was identical to the MLC strain.

The E. coli isolated from tissue homogenate of the scouring control animal was further examined for its pathogenicity. A broth culture of this hemolytic E. coli was fed to a newborn pig and it died within 72 hours due to scouring.

Bacteroides counts (Table 17) were always higher in the homogenate of the scouring pig, but the intestinal contents of the experimental pig contained more Bacteroides sp. than the scouring control animal.

Feeding Concentrates of Streptococcus diacetilactis

The cultural properties of the S. diacetilactis strain used appear in Table 18. It can be seen that the organism possessed all the characteristics of the genus Streptococcus and of S. diacetilactis in particular. It should also be noted that the organism was highly sensitive to acid pH conditions; however, the organism was not sensitive to 2% sodium taurocholate, an important colonization characteristic according to several workers (Speck et al., 1973; Rettger et al. 1935).

Before feeding, serial dilutions of the concentrate were plated on lactic agar and the total count was found to be 2×10^{10}/ml. Results of the feeding experiment are given in Table 19. From the

Table 18. Characteristics of the Streptococcus diacetilactis organism used in the Study.

Test	Results
Morphology	Gram positive cocci
Growth at 10 C	+
Growth at 45 C	-
Coagulation of milk in 48 hr at 22 C	+
Diacetyl produced in milk	+
Ammonia from arginine	+
CO_2 from citrate	+
Differential broth[a]	Acid, CO_2 and NH_3
2% sodium taurocholate	Not sensitive
Acid tolerance at pH 2.0, 3.0 and 4.0	Sensitive

[a] Reddy et al. 1970.

Table 19. Effect of Feeding a Concentrate of Streptococcus diacetilactis on Fecal Coliform and Lactobacilli Counts x 10^8/gm in Swine.

Test Pigs[b]		Control Pigs[b]	
Coliforms	Lactobacilli	Coliforms	Lactobacilli
11.80	10.60	14.60	13.80
9.50	10.00	12.00	13.30
7.80	8.90	4.60	12.00
8.70	7.40	2.60	9.60
6.30	9.60	2.30	7.90
5.90	8.10	2.70	8.80
5.90	10.90	4.50	6.80
4.40	8.60	5.00	8.90
5.30	9.50	3.00	8.20

[a] No S. diacetilactis organisms were recovered.

[b] Average data of four pigs.

results it can be seen that there was no significant reduction in coliform counts as compared to the control animals, nor was there much change in the fecal Lactobacillus counts. In spite of feeding large quantities of concentrate, no S. diacetilactis organisms were recovered from fecal samples when plated on lactic agar.

Bacterial Colonization of the Intestines of Pigs

As reported by many workers, the intestines of a newborn pig are sterile; data from the present study also suggested that bacterial colonization was absent in a newborn pig. This can be seen in Figure 12. No bacteria were seen either in the lumen or in the villi of the small intestine.

Colonization Pattern in Scouring Pigs

Results obtained were in agreement with reports cited in the review of literature on colonization by enteropathogenic E. coli in scouring pigs; a large number of coco-bacillary shaped, Gram-negative organisms were seen colonizing not only the mucus layer but also throughout the nine parts of the small intestine of a scouring pig. This is shown in Figures 13 and 14.

Colonization Pattern in Non-Scouring Pigs

With regard to the bacterial colonization pattern in control

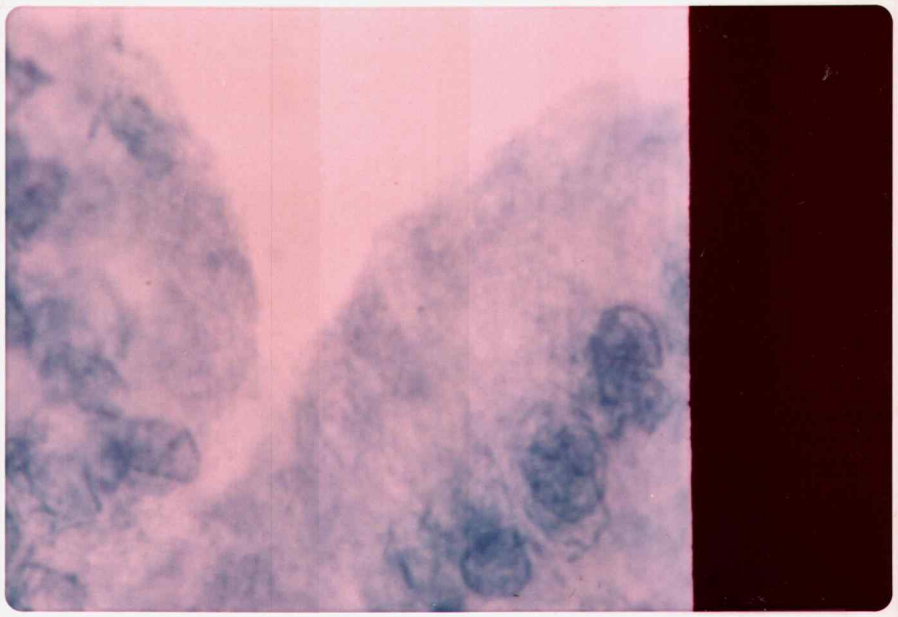


Figure 12. Photomicrograph of a Toluidine Blue Stained Section of the Intestine Taken from a Newborn Pig.

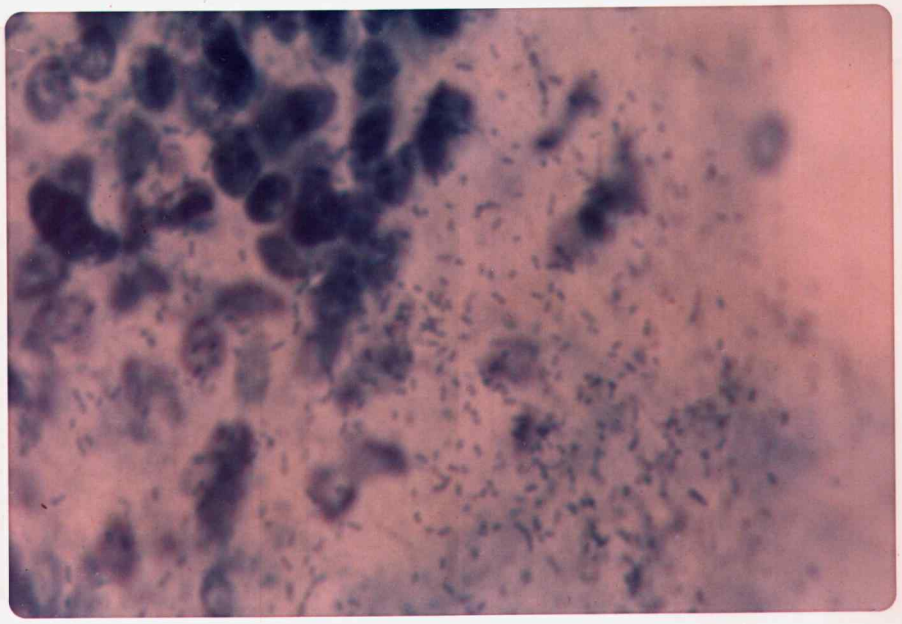


Figure 13. EEC Distributed on the Mucus Layer of Toluidine Blue-Stained, Frozen Sections of the Small Intestine from a Scouring Pig.

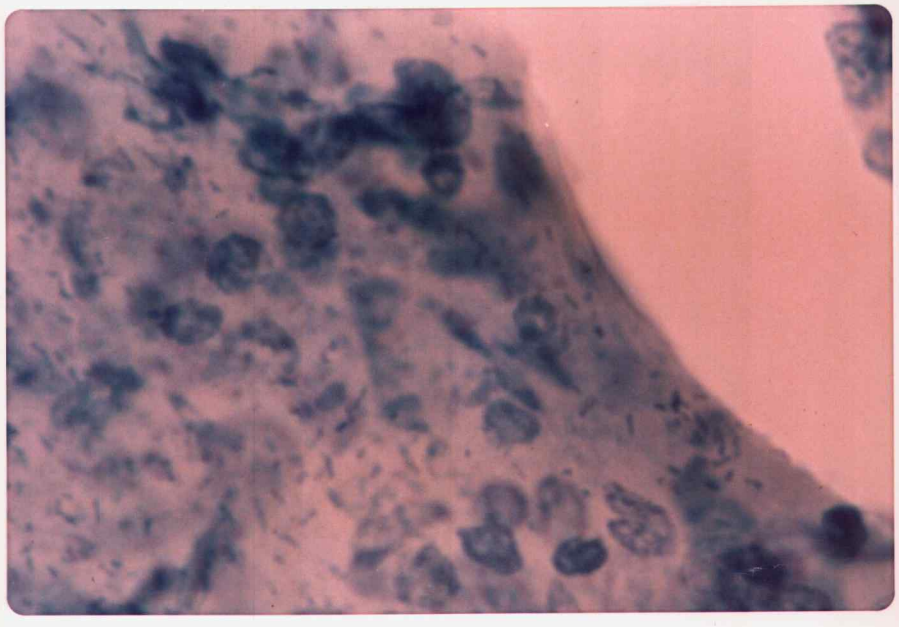


Figure 14. EEC Distributed on the Villus Showing Attachment to Epithelial Cells of a Toluidine Blue-Stained, Frozen Sections of the Small Intestine from a Scouring Pig.

non-scouring animals, the importance of balance between desirable and undesirable bacteria was again demonstrated. Here, even though some Gram-positive cocci and a few coco-bacillary organisms were seen, large numbers of Gram-positive rod-shaped bacteria (lactobacilli) were found colonizing most of the small intestine (Fig. 15 and 16).

Influence of Feeding Lactobacillus MLC Concentrates on Colonization Pattern

Results from the previous experiments indicated that the Lactobacillus organisms fed colonized the small intestines of the pigs and prevented colonization by EEC. Therefore further experiments were carried out to obtain more direct evidence for colonization by the Lactobacillus and its role in the prevention of diarrhea.

Frozen sections of the intestines from pigs fed concentrates of the MLC strain revealed (Fig. 17) a large number of Gram-positive bacilli colonizing the mucus layer and distributed throughout the body of the villus. No lactobacilli were seen inside the epithelial cells instead they were adhering to the surface. These pigs were kept in a clean place and were fed sterile milk so that colonization by other organisms was reduced. However, the control pigs which were maintained under similar conditions (except that they were not fed Lactobacillus organisms) revealed a large number of coco-bacillary E. coli organisms colonizing throughout the small intestine.

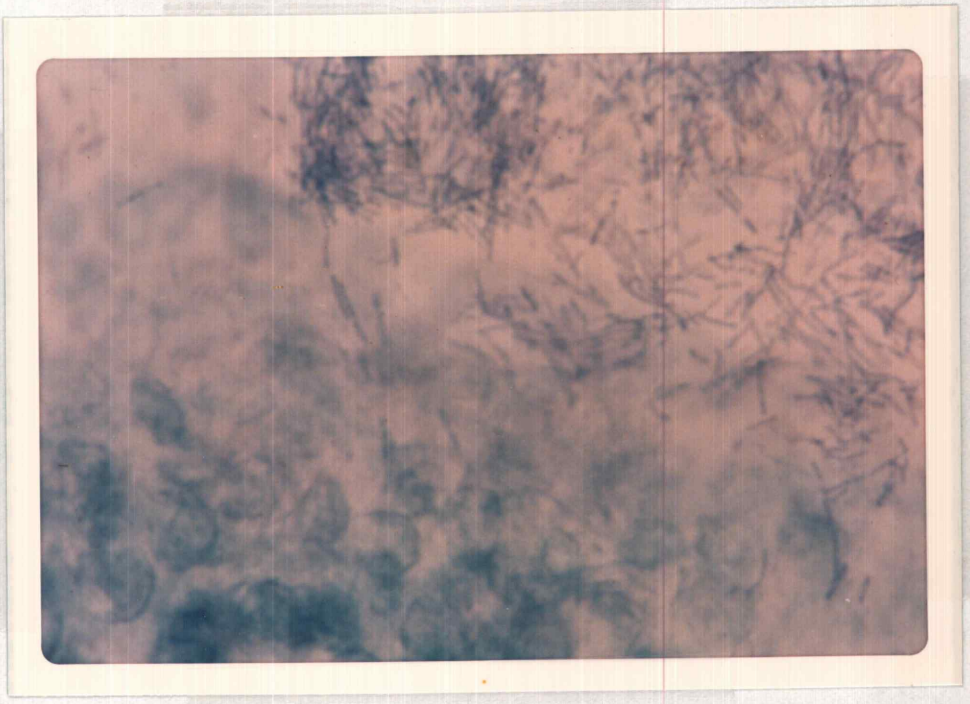


Figure 15. Micrograph of a Frozen Section of the Small Intestine from a Non-Scouring Piglet Stained with Toluidine Blue, Showing a Large Number of Lactobacilli Colonizing.

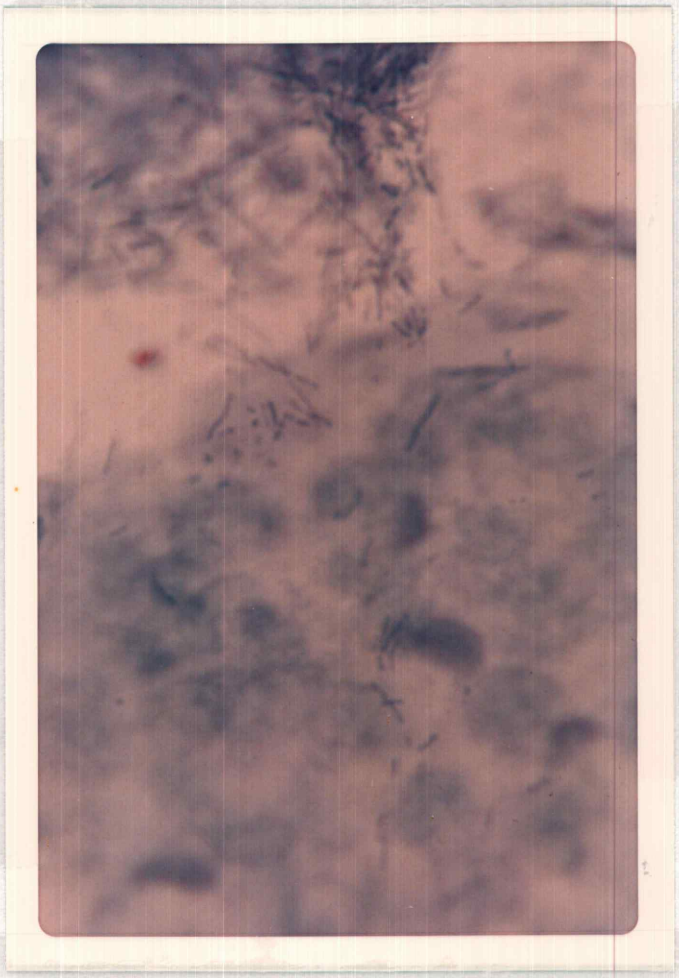


Figure 16. Micrograph of a Frozen Section of the Small Intestine from a Non-Scouring Piglet Stained with Toluidine Blue, Showing Very Few E. coli and Cocci but Large Numbers of Lactobacilli.

Figure 17. Gram Stained Frozen Sections of the Small Intestine from Lactobacillus-Fed Piglets Showing Large Number of Lactobacillus MLC Colonizing the Villi.

In addition, the control pigs died at about 36 hours, whereas the test pigs were still healthy despite never having received any colostrum. Figure 18 shows the colonization pattern in control pigs.

Effect of Various Intrinsic and Extrinsic Factors on Colonization by EEC and Lactobacilli in Pig Intestine

Colostrum is required by most mammals, especially pigs for postnatal survival. It is believed to inhibit EEC either by means of iron-binding proteins or immunoglobulins, thus providing passive immunity to the newborn. In vitro tests revealed that colostrum from sows did not have sufficient iron-binding proteins (lactoferrin and transferrin) to account for its inhibitory activity against EEC. This was in agreement with other reports on iron-binding proteins in sow's milk (Masson and Heremans, 1971). For this reason it was decided that immunoglobulins present in the colostrum probably were responsible for the inhibition. Colostrum derives its immunoglobulins from the blood. Therefore, it was reasoned that serum from a lactating sow might influence colonization by E. coli and lactobacilli. Groups II, III and IV animals showed various degrees of colonization by lactobacilli (Fig. 19 and 20) and Table 20 summarizes the observed colonization pattern in the different groups of animals. It is clear that the Lactobacillus-fed animals were colonized by a large number of lactobacilli which prevented colonization by other bacteria. Even though pigs in group V received Lactobacillus MLC organisms along

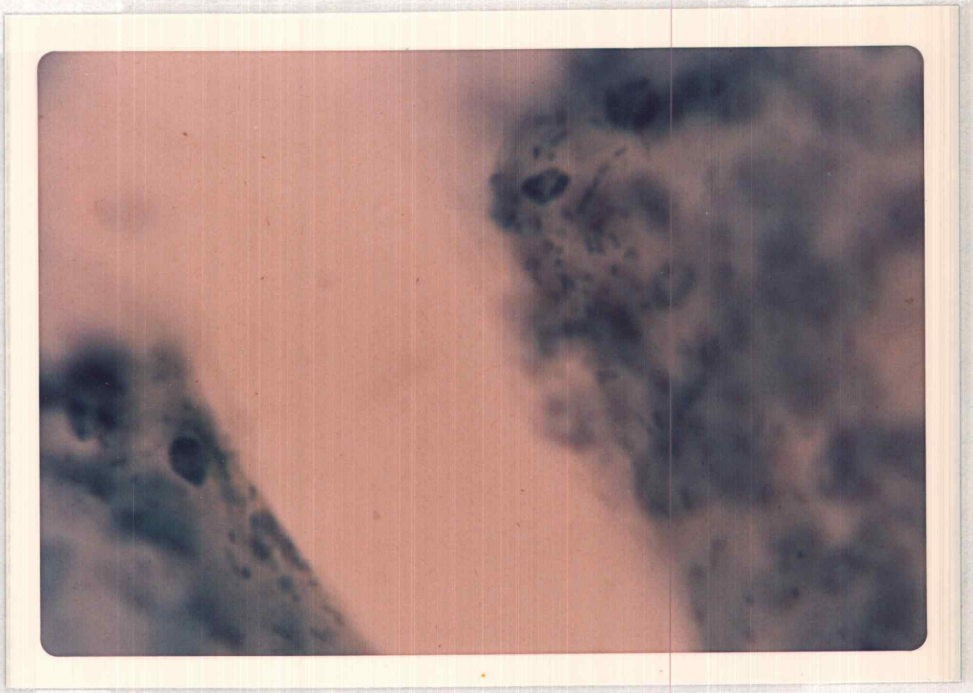


Figure 18. Micrograph of a Frozen Section of the Small Intestine from Control Pigs, Showing Large Number of EEC Colonizing the Villi.

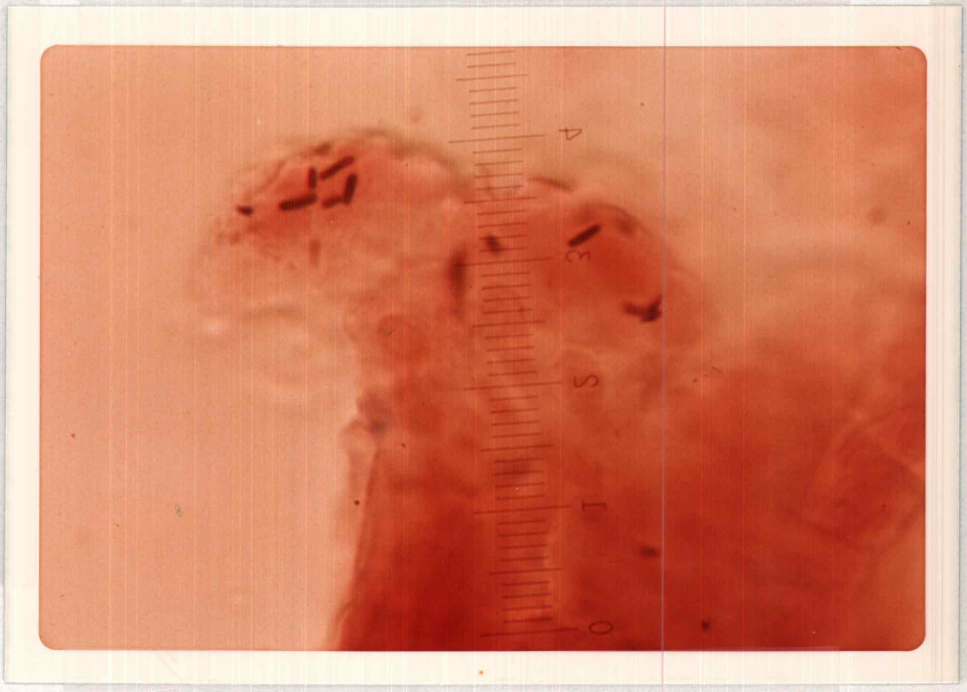


Figure 19. Gram-Stained Frozen Sections of the Small Intestine from a Pig Fed Lactobacillus MLC Showing a Villus with the Colonizing Lactobacilli.

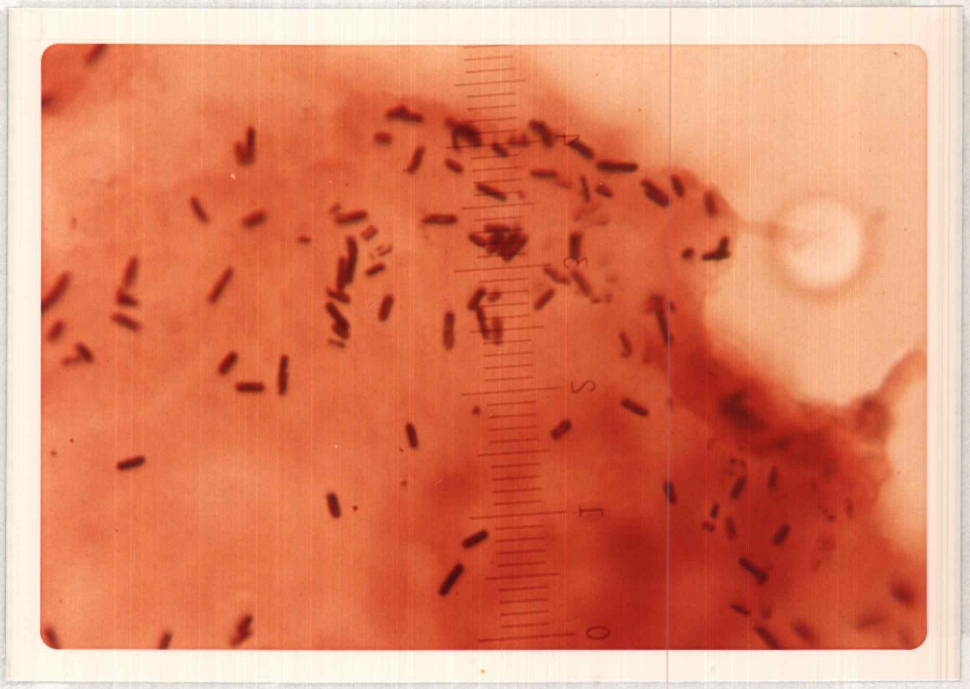


Figure 20. Gram-Stained Frozen Section of the Small Intestine from Pig Fed Lactobacillus MLC Showing Colonizing Lactobacilli.

Table 20. Results on Colonization of Organisms in Frozen Sections of Parts I through IX of the Small Intestine Before EEC Challenge as Revealed by Gram and Toluidine Blue-Staining

Group	I	II	III	IV	V	VI	VII	VIII	IX
I									
L.R.S.[a]	-	-	-	-	-	-	-	-	-
C.B.[b]	+++++	++++	+++++	+++++	++++	++++	++++	++++	++++
C.[c]	-	+++	+++	++	+++	++	++	+++	++
II									
L.R.S.	++++	++++	+++	++++	+++	+++	+++	+++	+++
C.B.	-	-	++	+	++	+	++	++	++
C.	-	-	+	-	-	-	+	++	++
III									
L.R.S.	++++	++++	++++	++++	+++	+++	+	++	++
C.B.	-	-	-	-	++	-	++	-	+
C.	-	-	-	-	++	-	+++	++	++
IV									
L.R.S.	+++	++++	++++	++++	+++++	++++	+++	+++	+++
C.B.	+	-	-	-	-	+	-	+	+
C.	-	-	-	-	-	-	++	+++	++
V									
L.R.S.	+	++	+	++	-	+	+	+	-
C.B.	+++++	+++++	++++	++++	++++	++++	++++	++++	-
C.	Tissue Destruction		+++	++++	++++	++++	+++	++++	-

[a] L.R.S. - Long Rod Shaped Form

[b] C.B. - Cocco-Bacillary Form

[c] C. - Coccus Shaped

with serum, they did not show many colonizing lactobacilli. Instead, a large number of coco-bacillary E. coli colonized and massive tissue destruction was evident. The serum fed had an agglutination titer of 1:20 and 1:40 against Lactobacillus MLC and EEC 09:K:NM respectively.

Bacterial colonization patterns after challenge with EEC 09:K:NM are shown in Table 21. Pigs in group I were given only EEC to check the virulence of the organism and a large number of coco-bacillary organisms colonizing throughout the small intestine were seen; these pigs died at about 36 hours. The second group of pigs which did not receive any colostrum started scouring at about 72 hours after the challenge with EEC and frozen sections revealed a significant number of EEC, suggesting that colostrum was required in addition to the concentrates of Lactobacillus to prevent colonization by EEC and subsequent scouring. Pigs in group III and IV which received colostrum along with Lactobacillus organisms did not scour even 72 hours after the challenge with EEC and colonization by EEC was also very low. The pigs which received serum along with Lactobacillus (group V) were scouring even before the challenge with EEC and they died at about 36 hours; a large number of coco-bacillary organisms were seen colonizing all parts of the small intestine.

The scouring and mortality data appeared to be related to the

Table 21. Results on Colonization of Organisms in Frozen Sections of Parts I through IX of the Small Intestine After EEC Challenge as Revealed by Gram and Toluidine Blue-Staining.

Group	I	II	III	IV	V	VI	VII	VIII	IX
I									
L.R.S.[a]	-	-	-	-	-	-	-	-	-
C.B.[b]	++++	++++	++++	++++	++++	++++	++++	++++	++++
C[c]	-	+++	+++	++	+++	++	++	++	+++
II									
L.R.S.	++++	++++	++++	+++	+++	+++	+++	+++	+++
C.B.	+	++	++	+++	+++	+++	+++	+++	+++
C	+	+	+	+	+	+	+	+	+
III									
L.R.S.	+++	++++	++++	++++	++++	++++	++++	++++	++++
C.B.	-	-	++	++	++	++	++	+	++
C	-	-	-	++	++	++	++	+	+
IV									
L.R.S.	++++	++++	++++	++++	++++	++++	++++	++++	+++
C.B.	++	+	+	+	+	++	+	+	+
C	++	++	+++	+++	+++	++++	++	++++	+++
V									
L.R.S.	+	+	+	-	-	-	-	-	-
C.B.	++++	++++	++++	++++	++++	++++	++++	++++	++++
C	++	+++	+++	+++	+++	++	+++	++++	++++

[a] L.R.S. - Long, Rod-Shaped
[b] C.B. - Coco-bacillary-Shaped
[c] Coccus-shaped

colonization by E. coli and Lactobacillus organisms. Table 22 shows the scouring results. Group I animals, which started scouring and died at about 36 hours, and a large number of coco-bacillary EEC. Group II, III and IV animals which did not scour up to the time they were sacrificed (72 hours) showed very few colonizing EEC but a large number of lactobacilli. Group V animals, however, reacted similar to the control group in spite of receiving the concentrate.

To get conclusive evidence that the Lactobacillus MLC fed was colonizing in the small intestine, FA staining was used. Better results were obtained by using the indirect FA technique than the direct method. Frozen sections obtained from the Lactobacillus MLC-fed pigs were stained and results obtained are shown in Figures 21, 22 and 23. From the figures, Lactobacillus organisms can be seen colonizing the villi in large number. From this it was clear that Lactobacillus MLC when fed to pigs in a concentrate form was able to colonize the small intestine.

The results of the study on the effect of postnatal feeding of Lactobacillus MLC concentrate on fecal coliform and lactobacilli counts are presented in Tables 23, 24 and 25. Since there was too much variation between pigs in different litters, it was not possible to average the data. Nevertheless, from the results it can be seen that the concentrate did not affect the fecal bacterial flora. This was probably due to the limited feeding (3 exposures) which was done.

Table 22. Scouring and Mortality Results Before and After Challenge of Piglets with EEC 09:K:NM.

Group	Test	Survival hr[a]	Scouring at 72 hr	
			Before Challenge[b]	After Challenge
I	NFM[c]	36	+	NA
II	NFM + Lactobacillus MLC	72	-	+
III	NFM + Lactobacillus MLC + colostrum	72	-	-
IV	Lactobacillus MLC; with the sow	72	-	-
V	NFM + Lactobacillus MLC + serum[d]	36	+	NA

[a] After 72, one animal per group was sacrificed.

[b] Saline suspension of EEC 09:K:NM.

[c] NFM - Non fatmilk.

[d] 1:20 EEC antibody titer.

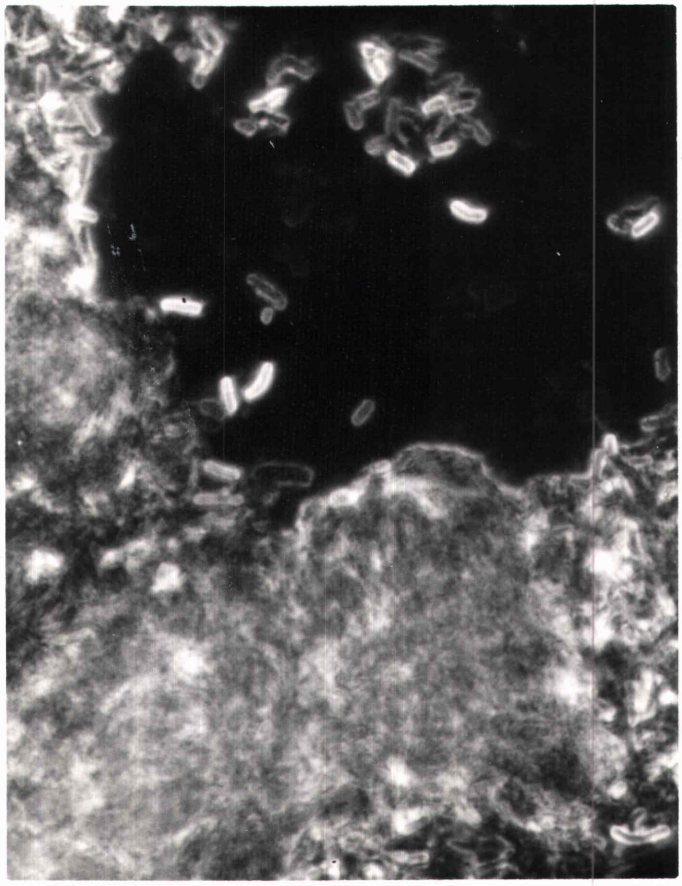


Figure 21. Fluorescent Antibody Stained Section of the Small Intestine from a Lactobacillus-Fed Pig Showing the Colonizing Lactobacillus MLC.

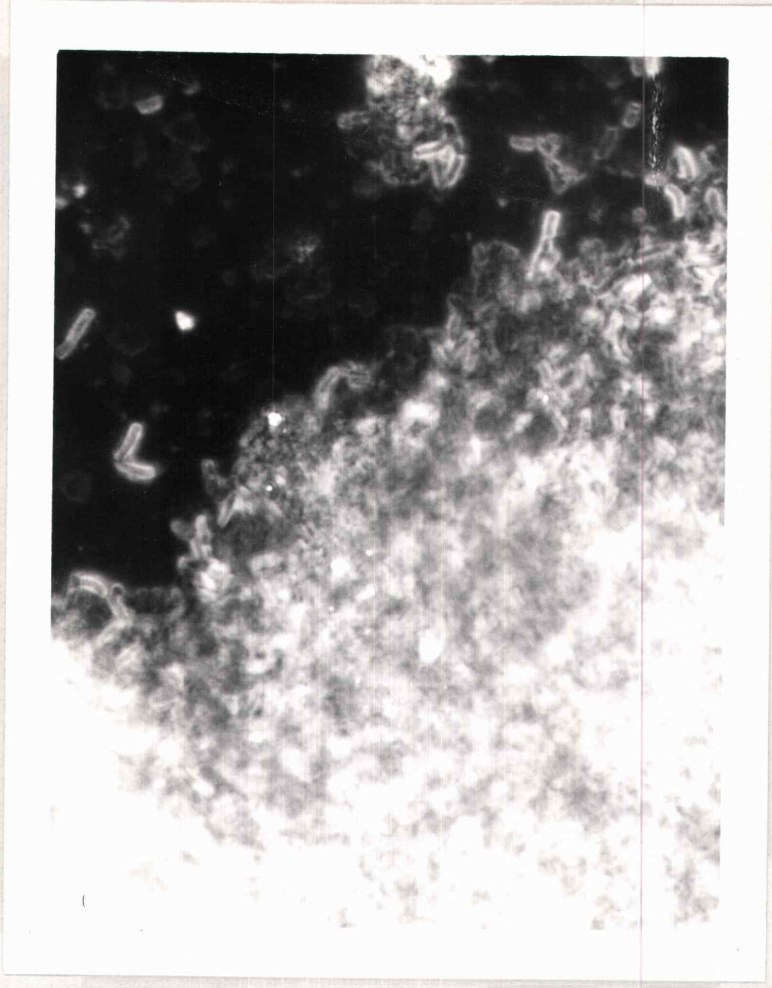


Figure 22. Fluorescent Antibody Stained Section of the Small Intestime from a Lactobacillus-Fed Pig, Showing Colonizing Lactobacillus MLC.

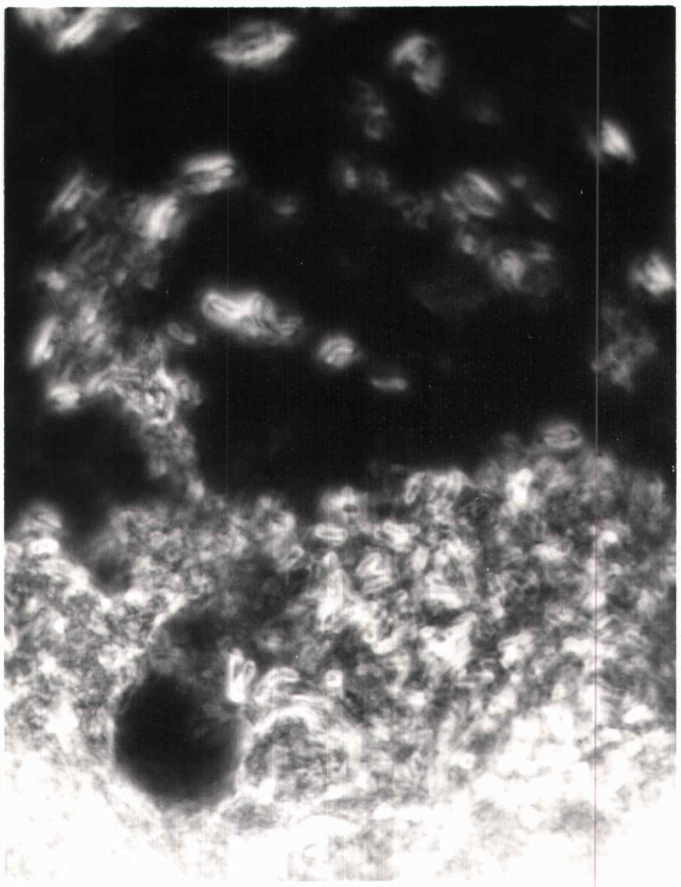


Figure 23. FA Stained Section of the Small Intestine from a Lactobacillus-Fed Pig Showing Colonizing Lactobacillus MLC.

Table 23. Average Fecal Bacterial Counts from Control and Experimental Litters.

Control Pigs X 10^8 cfu/gm		Experimental Pigs[a] X 10^8 cfu/gm	
Coliforms	Lactobacilli	Coliforms	Lactobacilli
0.02	0.11	0.03	0.3
0.22	2.70	8.00	10.0
0.19	1.00	2.70	3.1
0.32	3.00	2.90	25.00
-	-	15.00	25.00

[a] Received Lactobacillus MLC concentrate at 0, 12 and 24 hours after birth.

Table 24. Average Fecal Bacterial Counts from a Control and Experimental Litter.

Control Pigs X 10^8 cfu/gm		Experimental Pigs[a] X 10^8 cfu/gm	
Coliforms	Lactobacilli	Coliforms	Lactobacilli
0.0033	28.00	13.00	390.00
0.49	63.00	10.00	460.00
0.45	53.00	14.00	320.00
0.27	88.00	14.00	390.00

[a] Received Lactobacillus MLC concentrate at 0, 12 and 24 hours after birth.

Table 25. Average Fecal Bacterial Counts from a Control and Experimental Litter.

Control Pigs X 10^8 cfu/gm		Experimental Pigs[a] X 10^8 cfu/gm	
Coliforms	Lactobacilli	Coliforms	Lactobacilli
6.00	50.00	10.00	180.00
0.29	57.00	3.00	300.00
0.26	53.00	7.00	390.00
1.39	69.00	18.00	200.00
0.92	95.00	18.00	110.00

[a] Received Lactobacillus MLC concentrate at 0, 12 and 24 hours after birth.

In Vitro Inhibition Studies

Inhibitory Studies in Milk and Lactic Broth

The ability of Lactobacillus MLC to inhibit several pathogens during associative growth in broth cultures was studied and from the results (Table 26) it was clear that this organism was capable of inhibiting intestinal pathogens. Therefore, the inhibitory property was studied further.

Estimation of Acids Produced by Lactobacillus MLC

Due to the fermentative capacity of lactic acid bacteria, metabolic products, especially acids, must be considered as

Table 26. Summary of the Intestinal Organisms Inhibited by Lactobacillus MLC.

Organisms	% Inhibition at 24 hr[a]
β -hemolytic E. coli	99.99
S. aureus	99.99
Salmonella typhimurium	99.00
Bacteroides sp.	95.00
Clostridium perfringens	95.00
Streptococcus fecalis	95.00

[a] Compared to the test organisms growing alone.

potential inhibitors. The inhibitory properties of acids such as acetic, formic and lactic are well recognized and they have been implicated as agents responsible for bacterial inhibition in several studies. In this study, the Lactobacillus MLC strain was found to produce 0.38% lactic acid. No acetic, formic or propionic acid was found using GLC in either milk or lactic broth. The amount of lactic acid produced, however, was found to be inhibitory both to β -hemolytic E. coli and S. aureus. The pH of the broth when the MLC strain was grown in association with the indicator strain was 4.2. The pH of a 24 hour milk culture of the Lactobacillus MLC strain was 4.2 and the whey from this culture was inhibitory (no visible growth after 24 hours in broth culture) to S. aureus and β -hemolytic E. coli. To rule out the effect of pH in inhibition, a control tube

buffered at pH 4.0 with pH buffer tablets (Coleman Company) was tested for growth-supporting ability. The organisms grew well in the presence of pH 4.0 buffer.

Inhibitor Produced by Lactobacillus MLC

Isolation and Identification of the Inhibitor

The various fractions obtained during purification of the inhibitor were checked for protein, carbohydrate and inhibitory activity. The results are summarized in Table 27. After tentatively classifying the inhibitor as a carbohydrate, an alditol acetate derivative of

Table 27. Results of Tests for Inhibition, Carbohydrate and Protein Run on MLC Strain Whey Filterate and Fractionated Whey Filterate (see page 59). Inhibitory Activity Was Measured Against S. aureus.

Fraction	Inhibition	Anthrone	Ninhydrin
Supernatant whey	++++	++++	++++
1	++++	++++	++++
2	-	++	++
3	-	+	+
4	+++	++++	++++
5	+++	++++	-
6	+++	++++	-

the fraction was analyzed by GLC. This revealed three peaks, which corresponded to the retention times of galactose, glucose and 2-deoxy-D-glucose (Figure 24) in a standard preparation. Then a known standard of 2-deoxy-D-glucose was mixed with the sample and an alditol acetate derivative was similarly made and reanalyzed. Again, only the same three peaks were obtained, and also, the peak size of 2-deoxy-D-glucose increased as compared to glucose and galactose. These peaks corresponded to the alditol acetates of glucose, galactose and 2-deoxy-D-glucose, indicating the sugar was 2-deoxy-D-glucose.

Inhibitory Studies Using Pure 2-Deoxy-D-Glucose

The indicator organisms E. coli, S. aureus and S. typhimurium were inhibited both at pH 5.0 and 7.0 by 0.1% 2-deoxy-D-glucose. The Pseudomonas sp. was not inhibited at either pH levels. The results of the inhibition of S. aureus by 2-deoxy-D-glucose alone and in combination with the other two sugars are summarized in Table 28. The results indicated that 2-deoxy-D-glucose was a potent inhibitor alone as well as in combination with the other two sugars; actively growing cultures were inhibited to a greater extent than older cultures; the mechanism by which 2-deoxy-D-glucose is believed to inhibit bacteria has been reported (Biely et al., 1971).

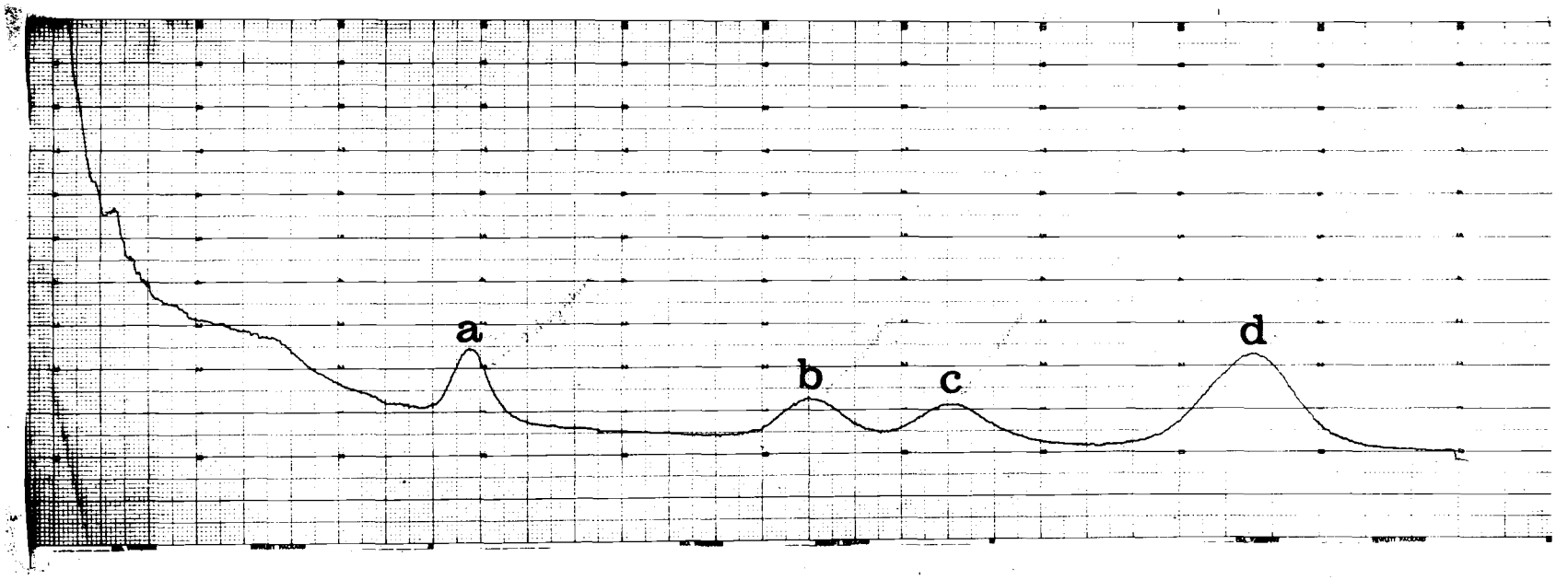


Figure 24. Photograph of the Recording Tracing Resulting from GLC Analysis of the Alditol Acetate Derivative of the Inhibitory Fraction Isolated from Lactobacillus strain MLC. From the left the four peaks, identified by corresponding in retention time to authentic compound, were 2-deoxy-D-glucose (a), galactose (b), glucose (c) and myo-inositol standard (d).

Table 28. Effect of Different Sugars on the Growth (Optical Density) of S. aureus in Modified Lactic Broth.

Time (hr)	Carbohydrate[a]							
	2-dg[b]	Glucose	Galactose	2-dg + Glucose	2-dg + Galactose	Glucose + Galactose	2-dg + Glucose + Galactose	None
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.005	0.01	0.00	0.005	0.00	0.005
8	0.00	0.025	0.02	0.01	0.00	0.01	0.005	0.01
12	0.005	0.02	0.035	0.015	0.005	0.03	0.005	0.02
16	0.005	0.08	0.08	0.035	0.005	0.09	0.025	0.04
20	0.00	0.16	0.14	0.07	0.00	0.17	0.06	0.08
24	0.00	0.29	0.17	0.12	0.00	0.26	0.10	0.16
36	0.00	0.49	0.25	0.36	0.04	0.43	0.25	0.21
42	0.00	0.50	0.27	0.42	0.14	0.45	0.30	0.24

[a] Sugars added at 0.1%.

[b] 2-deoxy-D-glucose.

Role of Antigen in Intestinal Colonization by Lactobacilli

Antigen Studies

In order to determine the possible role of cell surface antigens in colonization, it was necessary to determine the number of precipitin bands formed by the colonizing MLC strain when tested against its homologus antiserum. Antigen prepared from two other strains of lactobacilli (FHS and Lactobacillus salivarius) were then compared with the MLC strain to test for common antigens among the three species. Figures 25, 26 and 27 show the relatedness among the three species. L. salivarius did not give any precipitin bands when tested against MLC strain antiserum (Figure 25). Therefore, this strain was assumed to be a non-colonizing type in the feeding experiments. On the other hand, FH an autochtonous strain, was assumed to be a colonizing type. Figure 26 shows that the FH strain had three antigens in common with the MLC strain. Figure 27 shows the numbers of antigens seen in Lactobacillus MLC against its homologus antiserum.

Feeding Experiments

Concentrates of the above mentioned three strains of lactobacilli were then fed to piglets to test for ability to colonize the intestine. Table 29 shows the results; it can be seen that all three

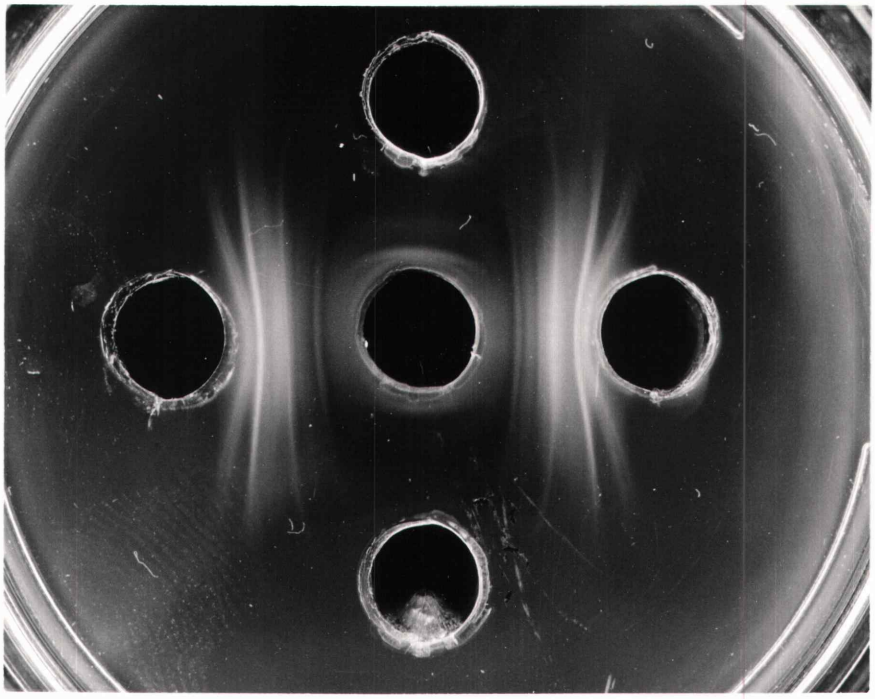


Figure 25. An Öuchterlony test showing the absence of antigenic relatedness between Lactobacillus MLC and L. salivarius. The center well contained MLC antiserum with homologous antigen in the left and right wells; the top and bottom wells contained L. salivarius antigen.

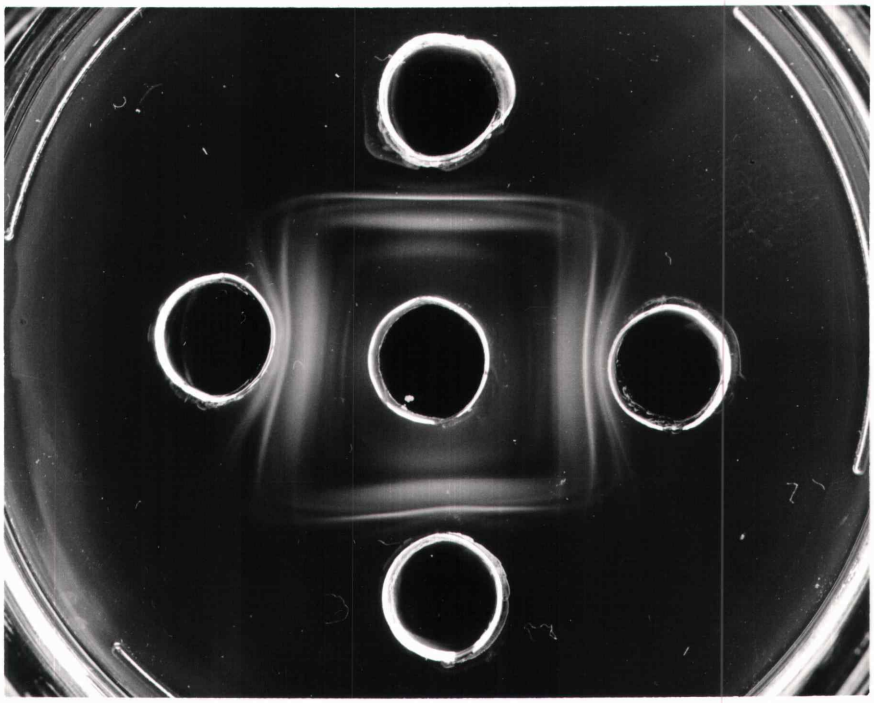


Figure 26. An Öuchterlony test showing the number of antigens common between Lactobacillus MLC and FH strain of Lactobacillus. The center well contained MLC antiserum with homologus antigen in the left and right wells; the top and bottom wells contained Lactobacillus FH antigen.

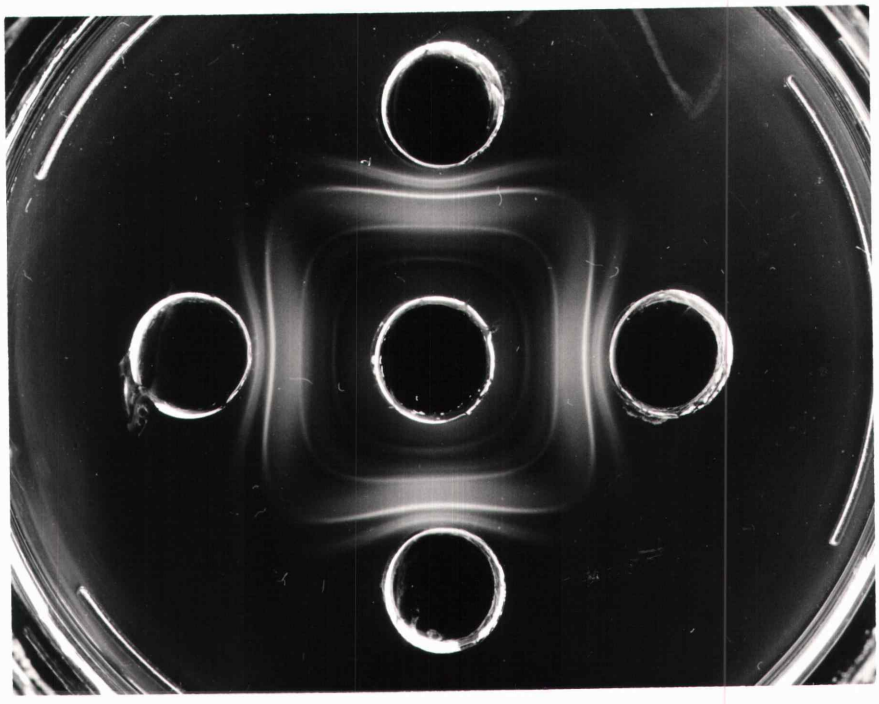


Figure 27. An Öuchterlony Test Showing the Number of Precipitin Bands Found in Lactobacillus MLC Antigen Against Its Homologus Antiserum.

Table 29. Results on Colonization of Organisms in Frozen Sections of Parts (I through IX) of the Small Intestine from Pigs Fed Various Lactobacillus Organisms.

Organisms Used		Parts of the Small Intestine								
		I	II	III	IV	V	VI	VII	VIII	IX
MLC Strain	LRS[a]	++++	+++	++++	+++	+++	+++	+++	+++	+++
	CB[b]	+++	+	++	+	+	+	++	++	++
	C[c]	++	++	++	++	++	+	++	+++	++
FH Strain	LRS	+++	+++	+++	++	+++	+++	+++	+++	+++
	CB	++	++	++	++	++	++	++	++	+
	C	++	++	++	++	++	++	++	+	+
L. salivarius	LRS	++++	+++	+++	+++	+++	+++	+++	+++	+++
	CB	++	++	+	++	+++	++	+++	+	+
	C	++	++	+	++	+	+	+	+	+
Control Pig	LRS	+	+	-	-	-	+	Not examined		
	CB	+++	+++	+++	++	++++	++++	Not examined		
	C	++	++	++	+++	++	+++	Not examined		

[a] Long rod shaped
[b] Coco-bacillary shaped
[c] Coccus shaped

strains colonized the small intestine of pigs. L. salivarius, which did not show any antigenic relatedness to the MLC strain, also colonized and to a similar degree as the MLC and FH strains. This indicated that antigen make-up was probably not involved in colonization by lactobacilli.

DISCUSSION

The majority of the organisms that colonize the alimentary tract of young animals are derived from their dams. One factor affecting the speed of colonization is the degree of contact between newborn animals and the source of colonizing organisms. Piglets which are active at birth soon come in contact with an environment highly contaminated by feces from the sow. This favors rapid entry by considerable numbers of bacteria into the alimentary tract, which leads to massive proliferation of E. coli, Clostridium perfringens and Streptococcus sp. in the stomach and flooding of these organisms into the small intestine. The low pH of the stomach contents is conducive to the growth of lactobacilli which become established as the principal component of the flora of the stomach and small intestine. This process of establishment of bacteria in the gastrointestinal tract is significant in the pathogenesis of infectious enteric diseases.

Considerable inaccuracy may occur in attempts to study and enumerate bacteria in material as bacteriologically complex as feces. However, the counts made daily from the feces of young pigs during this study were so consistent and followed such a definite pattern that there was justification for concluding that the behavior of some types of fecal bacteria was being studied with reasonable accuracy. Reassurance was also obtained from the fact that, where

comparisons were possible, counts of various types of bacteria in the pigs resembled in general those obtained by other workers using different techniques (Haenel and Muller-Beuthow, 1956).

In determining the normal bacterial flora of healthy non-scouring pigs, it was noted that the Lactobacillus counts were nearly always greater than the coliforms (E. coli) counts. On the other hand, in scouring animals, invariably the coliform counts were greater than the Lactobacillus counts. This is in agreement with work done by Chopra et al. (1963) who observed an increase in coliform and reduction in lactobacilli counts in scouring pigs. Dubos et al. (1963) also noted that lactobacilli were predominent in the intestinal tract of mice maintained under unusually clean conditions. This balance between lactobacilli and coliforms is sometimes altered due to unknown stress factors. During these times EEC may increase in numbers in the intestines resulting in colibacillosis and reduced animal growth. Due to the widespread use of antibiotics, the balance between intestinal lactobacilli and E. coli may be altered, causing serious colibacillosis problems.

This being the case, Lactobacillus organisms must have some beneficial effect in maintaining a healthy bacterial flora in the intestine. The treatment of intestinal disorders with lactobacilli is not a new idea. Metchnikoff (1908) believed that the genus was beneficial to man and animals. Rettger et al. (1921 and 1935) worked out

conditions necessary for the establishment of lactobacilli in the intestine. It also has been noted (Rettger et al. 1935) that a Lactobacillus must be a bile tolerant to become implanted in the intestine. However, in the work reported here, even though the organism used (L. lactis MLC) was inhibited by 2% sodium taurocholate, significant inhibition of coliform in the intestine occurred; this indicates that the property of bile tolerance is not a strict requirement for intestinal colonization.

There is confusion as to which species of Lactobacillus is most effective in intestinal disease therapy. Earlier reports (Herter and Kendell, 1908 and Rahe, 1915) indicated that L. acidophilus was effective and L. bulgaricus was not. The organism used in the present work was characterized using recognized, acceptable methods (Anonymous, 1968) including genetic analysis (Sriranganathan et al., 1973). The bacterium was not found to be L. acidophilus, but instead it was found to be L. lactis. Savage (1972) indicated that for an organism to become established in the intestinal tract it should be an autochthonous type, anaerobic and should colonize in the early life of the individual to become part of the indigenous flora. The organism used here grew better under anaerobic conditions but also grew well aerobically or without special attempts to maintain reduced aeration. It was first isolated from the human intestine.

In spite of feeding very large numbers of L. lactis MLC the

fecal lactobacilli counts did not increase significantly. This is in agreement with results obtained by Speck et al. (1973) and Paul and Hoskins (1972). The reason for this is not known; however, it may be that the Lactobacillus fed were colonizing in the small intestine, and only those which were in excess were voided in the feces. Alternately, the lactobacilli might have replaced the indigenous lactobacilli. It should be noted that the fecal coliform counts remained at the lower levels for 30 days after discontinuing the feeding of MLC Lactobacillus; this was indirect evidence that the lactobacilli fed colonized the small intestine and continued to exert their inhibitory effect on coliforms.

At this point in the study it was not certain what it meant in terms of animal health to reduce the fecal coliform counts to such a large degree. Intuitively, it seems that this should be of benefit. In this regard there have been recent reports indicating the requirements for intestinal pathogenicity of E. coli in swine. One of the most important is that the organism becomes localized in the upper part of the pig intestine (Gyles, 1972; Moon and Whipp, 1971; Bertschinger et al., 1972). In examining the microflora of severely scouring pig in different parts of the intestine, it was found that more hemolytic E. coli occurred in the tissue homogenate than in the lumen of the small intestine. This substantiated the results obtained by Smith and Hall (1968a) who found greater numbers of challenge

E. coli organisms in scrapings of the intestinal wall than in the contents of the small intestine. Thus, they inferred that the strain's ability to proliferate in the anterior small intestine was related to its ability to adhere to the epithelium--a property not possessed by non-pathogenic strains. Also, the diarrhea observed in the present study occurred during the period in which the numbers of E. coli in the small intestinal homogenate were maximal. This is compatible with the view that E. coli diarrhea results from local action of enterotoxin produced by bacteria on the intestinal wall (Smith and Halls, 1967a). Thus there was a real difference with regard to localization of hemolytic E. coli in the scouring and non-scouring control animals, suggesting that competition for colonization between lactobacilli and coliforms occurred.

During the recent American Society of Microbiology meetings Gorbach and Pierce (1973) reported that at least 10^5 EEC must be present per gram of intestinal tissue in order to produce typical symptoms of colibacillosis. On the other hand, Meyer (1972) has stated that as few as 50 toxin producing E. coli cells can cause the death of gnotobiotic pigs within 48 hours. The few number of organisms regarded in this latter case is no doubt related to the use of gnotobiotic pigs which do not have a normal indigenous flora, especially lactobacilli. In the present study by feeding concentrates of L. lactis MLC organisms, it was possible to reduce the E. coli to

less than 10^2/gm of tissue and from this it seems reasonable to conclude that such a feeding practice would be desirable as a preventive measure in colibacillosis prone swine herds. In view of this a satellite project to this research was undertaken (Sheggeby, 1974 - Thesis in preparation) in cooperation with the Animal Science Department. This was an effort to study, among other things, the influence of feeding L. lactis MLC concentrates on the incidence of colibacillosis in the O.S.U. Swine herd. While results were ambiguous, findings from the limited study clearly justified the need for continued research in this area.

Previous reports from this laboratory (Daly, 1972) on in vitro bacterial interactions have shown that S. diacetilactis organisms were able to inhibit E. coli up to 99.9% in broth cultures. However, in the present study, concentrates of S. diacetilactis did not have any inhibitory effect on coliforms in the intestines. The probable explanation for this is that this organism is sensitive to acid pH and unable to survive in the stomach. This is probably the reason for the non-recoverability of S. diacetilactis organisms from the fecal samples. This indicates that an organism must be acid tolerant to establish itself in the alimentary tract.

The findings of Kennworthy and Crabb (1963) that the intestinal tract of healthy pigs is sterile at birth was confirmed in the present study. Latter in life pigs come in contact with the surroundings

and colonization of the gastrointestinal tract by a variety of microorganisms results.

The bacterial colonization observed in healthy, non-scouring pigs in this present study emphasized again the importance the balance between lactobacilli and E. coli in maintaining a healthful conditions in the intestine. From earlier discussions on the fecal and tissue bacterial flora in scouring and healthy pigs, it is known that Lactobacillus counts are greater than E. coli counts in healthy swine and the reverse is true in a scouring animal. The present discussion deals with this balance at the tissue level. The presence of long rod shaped bacteria (lactobacilli) and a few cocci or cocobacillary shaped E. coli organisms was seen microscopically in healthy pigs. Though not much work has been done on the colonization by indigenous lactobacilli in the pig intestine and their role in the prevention of diarrhea, recent work with infants by Mata et al. (1972) has given clear evidence that colonization by bifidobacteria in breastfed children has resulted in a lower incidence of shigellosis and other enteric diseases.

There are many reports on the pathogenicity of E. coli. Takeuchi (1967) classified pathogenic intestinal bacteria into three groups according to their site of action in the intestines:

1. Pathogens that invade the intestinal mucosal layer.

2. Pathogens that attach themselves to the intestinal epithelium but are not invasive.

3. Pathogens that neither penetrate nor attach themselves to the intestinal epithelium but still produce symptoms.

The present work on bacterial colonization in pigs suffering from colibacillosis indicates that EEC attach themselves to the small intestinal villi and thus can be classified in Takeuchi's intermediate group. The bacteria were observed in large number on the free border of the villus epithelium and between adjoining villi. However, they were not grouped as invasive, since only occasionally organisms were seen within the epithelial cells. In addition to being located on villi, EEC were also observed at the apical region of intestinal crypts. They were rarely observed within the basal region of crypts; this may be due to the greater exposure of villi than crypts to EEC. However, the precise nature of the relationship between EEC and the intestinal epithelium can be determined only by electron microscopic studies. Staley et al. (1969) found that in starved newborn piglets, EEC is associated with the exfoliation of microvilli prior to the attachment of bacteria to the apical plasma membrane. On the other hand, Drees and Waxler (1970b) reported that the degeneration of microvilli was rarely observed and did not appear related to the presence of E. coli 0138:K81:NM, a non-fimbriated serotype.

The location of EEC on villi can be considered as a lesion of

coliform enteric disease in pigs. The results obtained by Arbuckle (1970) are in agreement with the above statement. He observed that numbers of EEC on villi were considerably greater than the numbers of non-EEC in a similar situation.

There is no direct evidence published to date as to the fate of lactobacilli fed to animals or human beings. All the reports published so far deal with numbers of cells found in fecal samples. However, as stated earlier, the fecal bacterial picture does not necessarily give a true picture of reactions happening at the tissue level. The only way by which this can be studied is microscopically. Another factor that should be mentioned is that for lactobacilli to be effective in maintaining a healthy intestinal flora, they have to colonize the small intestine. Again, the only way to look for colonization is by a direct observation. In the present study the indirect evidence of colonization by Lactobacillus MLC was confirmed by Gram and toluidine blue-staining of frozen sections. In contrast to colonization by EEC, the lactobacilli are more concentrated at the free border of the villi.

These colonizing lactobacilli probably inhibit the colonization by EEC and reduce the incidence of colibacillosis by two means:

1. By acting in competition with EEC for the same site on the intestine.

2. By a specific inhibitory effect on EEC and through a metabolic product, to be discussed later.

With regard to the first mode of action, the results presented in the present study provide support that competition for sites on the intestines is important since there were few or no EEC in areas colonized by lactobacilli. So the primary step in pathogenicity of EEC can be prevented by inhibiting colonization.

There are many reports describing the possible means by which EEC produce the symptoms of colibacillosis. Porter and Kennworthy (1969) attributed post-weaning diarrhea in pigs to the increased metabolic activity of E. coli in converting proteins to amines. Amines, being irritating and toxic, increase the intestinal peristalsis and thereby produce diarrhea. By feeding Lactobacillus organisms Hill et al. (1970) were able to reduce the fecal amine level and the incidence of diarrhea.

Changes in the oxidation-reduction potential often result in the dominance of particular organisms. The ability of lactic acid bacteria to lower the oxidation-reduction potential in their growth environment is well recognized and is probably a factor in their antagonistic relations against aerobic organisms. Even though E. coli is not a strict aerobe, the lowering of the oxidation-reduction potential and the resulting lack of oxygen may prevent E. coli from using the TCA cycle for energy. They would therefore be limited to glycolysis for their energy needs and thus, their activity and numbers become reduced.

Colostrum is known to benefit the postnatal health of all mammals. Results of the present study are in agreement with this fact. The possible role of the iron-binding proteins (lactoferrin and transferrin) present in colostrum in the inhibition of EEC in the gastrointestinal tract was studied; however, no inhibition was observed. This supported the results obtained by Masson and Heremans (1971) who found that colostrum from sows had only 20 to 200 μg/ml of iron-binding protein as compared to greater than 2 mg/ml in human colostrum. However, from this present study, sow's colostrum was shown to favor colonization by lactobacilli to a greater extent than EEC. Also, the symptoms of diarrhea were completely absent in colostrum-fed pigs. The reason for this are not known but it is noteworthy that Mata et al. (1972) observed that breast-fed children had a predominently stable Bifidobacterium flora. Also, Mata and Wyatt (1971) attach particular significance to the concentration of lysozyme and secretory IgA present in human colostrum where infant health is concerned. British scientists (Anonymous, 1973) have shown that the secretory IgA in sow's colostrum plays a major role in the prevention of the intestinal development of EEC. Gindrat et al. (1972) detected a high titer of antibodies presumably secretory IgA, in human milk against some of the most common serotypes of EEC. Smith and Halls (1968a) reported that antibody present in colostrum sensitizes invading E. coli to phagocytosis by cells of the reticulo-

endothelial system. Thus it appears that colostrum may favor colonization by lactobacilli and the presence of substances similar to the bifidus (Gyorgy and Rose, 1955) or carrot juice (Yoshioka et al. 1968) factors, may be playing an important role in this regard. More research is needed in this area.

Since colostrum receives all its immunoglobulins from the blood (Schlossman and Moro, 1903), it seemed logical to determine the influence of feeding serum from a newly lactating sow on colonization by EEC and L. lactis MLC. There are reports in the literature of success in prevention of E. coli infection by oral feeding of EEC antiserum to piglets (Smith and Linggood, 1971b; Smith, 1972). However, according to Fubura and Freter (1972), serum antibodies are rapidly degraded in the gastrointestinal tract due to the action of enzymes. It was found in the present study that feeding normal serum (agglutination titer of 1:20 against EEC 09:K:NM) neither prevented the colonization by EEC nor favored the colonization by Lactobacillus MLC. Instead, pigs began scouring in spite of receiving lactobacilli. These results supported the report made by Fubura and Freter (1972). The present study also showed that colostrum was required for the postnatal survival of piglets. Even though colostrum immunoglobulins are derived from blood, it should be noted that serum IgA is different from secretory IgA present in colostrum. The secretory epithelial cells add a secretory protein

portion to the serum IgA as it passes through the secretory epithelial cells. This additional protein protects the immunoglobulins from bacterial and host enzymes in the gastrointestinal tract. Thus the immunoglobulins in the colostrum are more protective in the gastrointestinal tract than the serum immunoglobulins.

To the author's knowledge there are no known reports on the use of the FA technique to study intestinal colonization by lactobacilli. Lactobacilli are weak antigens, so repeated immunizations are required to produce a sufficiently high antibody titer to be used in the FA technique. The antiserum was fairly specific but probably could be improved by reducing the duration of immunization; the antiserum produced against the MLC strain cross reacted with L. acidophilus, L. bulgaricus and L. lactis ATCC 12315. These three lactobacilli are closely related in their biochemical, cultural and genetic characters. It was also found that these three organisms were closely related antigenically as determined by the Öuchterlony test.

From results obtained with the FA technique, it was possible to say conclusively that Lactobacillus MLC when fed to piglets, colonized the small intestine and reduced the colonization by other enteric pathogens. The FA technique was useful for studying the fate of Lactobacillus organisms fed, since it could be used to stain tissues as well as fecal specimens.

Results obtained when challenge dose of virulent EEC 09:K:NM

was given to L. lactis MLC fed pigs were encouraging. The inability to produce the symptoms of diarrhea even 72 hours after the challenge dose (control animals died at 36 hours after experimental infection) indicated a definitive protective role played by L. lactis MLC. However, pigs which did not receive colostrum but only lactobacilli showed symptoms of diarrhea at about 72 hours. Even though L. lactis MLC colonized in this group of pigs, there were a few EEC also colonizing. In contrast, animals in the other group which received colostrum and lactobacilli (MLC) showed no signs of diarrhea. This again signified the importance of colostrum in the postnatal health of pigs.

There are many reports on the mechanism of intestinal colonization by EEC. The K88 fimbrial antigen is found exclusively on E. coli strains enteropathogenic for swine. Recently, it has been shown that the K88 fimbriae play an important role in the colonizing ability, and hence the enteropathogenicity of E. coli strains in pigs (Smith and Linggood, 1971a). However, no research has been done on the mechanism of colonization by lactobacilli. In the present study, the role of surface antigens in lactobacilli (similar to the K88 fimbrial antigen) in colonization of the intestines was studied. From the study it can be stated that such antigens probably are not involved in the colonization process. However, more research on the various antigen fractions obtained and their role in colonization is needed.

Recently Takeuchi and Savage (1973) reported that only certain lactobacilli can attach to the gastric squamous epithelium in the mucosa. This specificity they suggested resides in a particular acid mucopolysaccharide substance produced by the bacteria.

Mitsuoka (1971), reporting on implantation of lactobacilli in the intestine, stated that a non-intestinal strain could not become established in the intestine and, even among the intestinal strains, species specificity is exhibited. This work was done using germ-free chicks, in which the antibody-forming system does not function well. Morishota et al. (1971) reported that L. acidophilus ATCC 4356, a human intestinal strain, failed to become established in the intestines of chicks. This was again attributed to some sort of host specificity. However, the method used by these workers to study the colonization (fecal counts) were very indirect. This is in contrast to the results obtained in the present study where no species specificity was exhibited. L. salivarius (non-intestinal origin) and L. lactis MLC (human intestinal type) colonized to the same extent as the autochthonous pig strain (FH) in the pig intestine.

Lactic acid bacteria are known to produce a variety of substances which are bactericidal, including some antibiotics. For example, recently an antibiotic from L. acidophilus isolated by Vakil and Shahani (1965) was patented. In the associative growth experiments in milk and lactic broth, the pH generally dropped to

4.2. The attainment of this low pH value suggested that acid production is a possible mechanism of inhibition. Test organisms would not grow in cell-free supernatants (whey) of Lactobacillus MLC in milk and lactic broth cultures. It was observed that the organism was homofermentative and did not produce any short-chained fatty acids such as acetic, propionic or butyric acids. The only acid produced was lactic acid (0.38%). However, the test organisms grew at pH 4.0 in lactic broth, indicating that some factor(s) other than acids was responsible for the inhibitory activity.

On further analysis, it was found that the inhibitory culture supernatant (milk culture) contained 2-deoxy-D-glucose in addition to glucose and galactose. Further work using authentic 2-deoxy-D-glucose showed that it inhibited the test organisms. Actively growing cultures of the test organisms were more susceptible than inactive cultures and microscopic examination of the treated cultures of test organisms revealed cell walls of lysed bacteria. Bieley et al. (1971), in their study on the effect of 2-deoxy-D-glucose on cell wall formation in Sacchromyces cervisiae, stated that phosphorylated derivatives of 2-deoxy-D-glucose interferred with those processes of yeast metabolism in which the corresponding metabolites of glucose and mannose take part or are formed. Thus, the processes most affected by 2-deoxy-D-glucose metabolites should be those leading to synthesis of cell wall polysaccharides. Also Murer (1969) has shown that

2-deoxy-D-glucose inhibits the glycolytic pathway in platelet metabolism. Since oxygen is limited in the gastrointestinal tract EEC are likely limited to growth by glycolysis and therefore sensitive to 2-deoxy-D-glucose produced or present in Lactobacillus MLC concentrate.

From the limited data reported in this study, it would appear that by feeding concentrates of L. lactis MLC, the E. coli counts both in fecal sample as well as in the intestinal tissue and the lumen will be reduced. Thus, a balance will be maintained between the desirable and undesirable bacteria, which is required for a healthy intestinal tract. This reduces the incidence and severity of scouring.

The application of this work in the swine industry would appear to be of tremendous value. The increased number of antibiotic-resistant EEC has limited the possibility of indefinite and indescriminate use of antibiotics in both human infants and pigs. Therefore, application of a natural process of preventing or treating disease should be used to a greater extent. England et al. (1972), while comparing the efficacy of some techniques to substitute for the use of antibiotics in the pig industry, discusses the advantages of use of lactic acid bacteria as compared to other methods. Larousse (1970) reported that the addition of L. acidophilus to poultry feeds resulted in effects similar to those obtained from antibiotics including superior effects to antibiotics as growth stimulants.

Now, the question arises as to how bacteria can best be fed to a large number of animals. Feeding through the drinking water system may not be efficient since nursing piglets do not drink much water until they are about 2 to 3 weeks old and so they would not take in the required number of lactobacilli. Another possibility would be the use of lyophilized organisms as food additives; again, nursing piglets may not get much food and the viability of the lyophilized organisms may be decreased. A third and most feasible method would be to bottle-feed newborn piglets. This can be done at the time when the piglets receive their oral dose of iron or other nutrients. Since, the organisms fed act as antigens, they should be available before the immune system of the piglet starts functioning (about 2 weeks); therefore, lactobacilli, when fed to nursing piglets before their antibody-producing system develops helps in establishing a proper intestinal flora. Another question is how long should the pigs be fed. In the experiment where the piglets were fed at 0, 12 and 24 hours of age, not much benefit was seen. This indicates that piglets should be fed for a longer period, probably each day for a week or two or until the immune system start functioning. From the histological studies it was shown that when piglets were fed Lactobacillus MLC every 2 hours for the first 24 hours and every 4 hours for the next 48 hours, the bacteria colonized the small intestine, prevented colonization by EEC and prevented colibacillosis.

SUMMARY

The concentrate strain of Lactobacillus MLC used in feeding experiments was characterized using biochemical, serological and genetic characters. By feeding concentrates of this organism, it was possible to reduce the fecal coliform counts up to 99.9%, the incidence and severity of scouring was also reduced. The feasibility of mass feeding was studied by supplying the organisms through the drinking water system; similar results were obtained. It also was possible to reduce the EEC colonizing the intestinal tissue to less than the suggested minimum infective number (10^5/gm of tissue). Finally, by serological and biochemical tests the Lactobacillus isolated from the intestinal tissues of Lactobacillus-fed pigs was identified as the same strain fed.

Histological studies were then carried out to obtain direct evidence of colonization by the Lactobacillus fed. Gram and toluidine blue-stained frozen sections of the small intestine from Lactobacillus-fed pigs revealed many Gram positive bacilli colonizing the small intestine. Very few EEC colonized these treated animals but large numbers of EEC were found in the intestinal tissue from scouring pigs.

The influence of colostrum and normal serum from a lactating sow on colonization by Lactobacillus and EEC was studied.

Colostrum favored the colonization by lactobacilli. The effect of challenging pigs fed Lactobacillus MLC with EEC 09:K:NM showed that colonization by EEC and symptoms of colibacillosis were not evident in Lactobacillus-fed pigs. The colonization by Lactobacillus MLC was further confirmed by a more sensitive fluorescent antibody technique.

Feeding concentrates of S. diacetilactis to pigs did not reduce the fecal coliform counts nor were any S. diacetilactis organisms recovered from the feces.

The possible role of surface antigens in colonization by Lactobacillus MLC was investigated and findings indicated the absence of any antigen involvement.

Lactobacillus MLC was able to inhibit a variety of intestinal pathogens, apparently due to the production of 2-deoxy-D-glucose.

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