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THE ANTIMICROBIAL ACTIVITY OF GANGLIOSIDES ISOLATED FROM LACTIC ACID BACTERIA, CULTURED DAIRY FOODS, AND BOVINE MILK FAT

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Animal Science

2001

ABSTRACT

THE ANTIMICROBIAL ACTIVITY OF GANGLIOSIDES ISOLATED FROM LACTIC ACID BACTERIA, CULTURED DAIRY FOODS, AND BOVINE MILK FAT

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There is substantial evidence suggesting that lactic acid bacteria (LAB) have an inhibitory effect on pathogenic bacteria in the intestinal tract. LAB and LAB used for manufacturing cultured dairy foods have been shown to modify the metabolic activities of the colonic microflora, possess antimicrobial activity, and neutralize *Escherichia coli (E. coli)* enterotoxin. Although various mechanisms have been proposed for these effects, the involvement of LAB gangliosides contained in these products was the focus of this research.

Mixed gangliosides were extracted and purified from sweet and cultured buttermilk, LAB, and yogurt. These products were targeted because it is known that they contain gangliosides. Using an agar overlay plate technique, mixed gangliosides recovered from these products demonstrated bacteriostatic effects, as evidenced by the retardation of *E. coli* growth around wells containing varying amounts of gangliosides. Mixed gangliosides have also been demonstrated to inhibit mannose-resistant pilus-mediated adhesion of *E. coli.* The hemagglutination-inhibition assay results suggest that gangliosides may limit the adhesion of enterotoxigenic *E. coli* to the intestinal mucosa and thus prevent diarrhea. The application of this research to *Helicobacter pylori* (a bacterium strongly associated with gastric ulcers) was demonstrated by inhibition of

pilus-mediated adhesion of this organism as determined by the hemagglutination-inhibition assay.

DEDICATION

To my mother, Mildred Boykins, my first teacher: Your patience and capacity for love I can only hope to emulate. The knowledge you have endowed me with will span my lifetime. You have made the education of others your life's work, and I am honored and fortunate to be counted among them.

To my father, James O. Boykins: By your example, you have impressed upon me the values, perseverance, and attitude needed not only to succeed, but truly to experience life. Through you, I have come to understand that the merit of one's life lies not in one's accomplishments, but in his or her positive effects on others and the community as a whole.

To my future husband, Michael A. Winrow: You have helped me unlock the mysteries of deoxyribonucleic acid, as well as the secrets of my heart. To my family and friends who have nurtured, encouraged, and believed in the dream; to the many educators who have provided me with the foundation needed to achieve the dream; but most of all to my parents, for making me a dreamer.

ACKNOWLEDGMENTS

I would like to express my gratitude to Dr. Melvin Yokoyama, my major professor, for training me in various microbial techniques, and for his guidance, support, encouragement, and friendship, all of which contributed to my completion of the doctorate degree.

I would like to thank Dr. Julius Jackson for rescuing me from the world of work and encouraging me to further my education at Michigan State University and, in the process, introducing me to my future husband. My debt to him will likely never be repaid.

I also would like to thank Dr. Sheila Grimes for her assistance, expertise, friendship, and positive attitude in the face of slow, hectic, and sometimes impossible odds.

I also wish to acknowledge the remaining members of my graduate committee, Dr. Steven Bursian and Dr. John Kaneene, for their advice and assistance.

My sincere appreciation is expressed to Dr. Kathleen Moore, whose expertise in chemical analysis allowed for the extraction of gangliosides, which was so crucial to this research, and to Dr. Anna Ettinger, whose preliminary work in this area provided fundamental data upon which much of our early research was based. They both were invaluable resources for information, consultation, and advice.

I would also like to express my gratitude to Darigold, Inc. (Mary Jens) for providing the powdered buttermilk and bacterial starter cultures for this research, to Michigan State University's Dairy Plant (Dr. John Partridge and Siggaard Torben) for

graciously supplying yogurt for ganglioside extraction, and Chris Hansen's Biosystems for supplying bacterial starter cultures.

I must acknowledge the help of Dr. James Jay, who believed that I could accomplish the task at hand. Without him, I might have given up long ago. He has personally assisted in adding to the ranks of those holding postgraduate degrees in science, and society's debt to him will likely never be repaid. My appreciation also is extended to Dr. Soegiarto for his endless assistance with the trials and tribulations of computers and their programs, Maggie Hoffman and Ralph Common for their graphics expertise, Dr. Robert Holland and Dr. Raul Hollingsworth for providing bacterial samples, and Dr. Sue Hengemuehle for providing everything that she could.

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CHAPTER I

LITERATURE REVIEW

For many years, scientists have identified the microbial world as an arch enemy of continuing healthful existence. For this reason, most researchers have emphasized the harmful effects of the microbial environment. Investigations dealing with the protective and regulatory role of normal microflora, however, recently have begun to increase.

Using "good" bacteria to fight or replace "bad" bacteria is far from a new concept, with research on this issue dating back more than a century. Metchnikoff's (107) research with cholera in the early 1900s usually is recognized as the earliest attempt at probiotic use. In his review, Florey (43) discussed work by Cantani in 1885, in which the replacement of *Bacterium tuberculosis* with a "harmless organism" was claimed. In 1916, Nissle used a bacterial strain called "Bacti. coli," which was made commercially available as "multiflor" for treatment of various intestinal problems.

Staphylococcus aureus strain 502A was used in the early 1960s to prevent intestinal colonization by *S. aureus* phage type 80/81, a virulent organism known to cause high rates of morbidity and mortality among neonates (8, 155). This experiment involved implanting the 502A strain in neonates before the more virulent *S. aureus* 80/81 strain could become established. Use of the 502A strain became fairly widespread. However, its use declined, partly because of decreasing need and also because the administration of

large doses of this strain was thought to be responsible for lesion development, which could lead to the disease itself (29).

More recently, other attempts have been made to block *S. aureus* infections in neonates; one such effort was Ehrenkrantz's implantation of diphtheroids and *S. epidermidis* (34). No harmful effects were produced, but the purpose also was not achieved. In 1973, Ludinova implanted a benign type 083 *Escherichia coli* in the gut of neonates to lessen intestinal colonization by bacteria and decrease the incidence of diarrhea (98). More recently, Rueda and colleagues (139) added gangliosides to an adapted milk formula and were successful in modifying the fecal flora of preterm infants. Tuomola and Salminen (174) demonstrated adhesive properties of probiotics and dairy lactobacillus strains. Implantation of bacterial strains is also being used for the prevention of dental caries (83, 89, 90) and urinary-tract infections (137, 166).

In 1989, Fuller (45) defined a probiotic as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance." This definition is somewhat limited, though, and a broader definition that does not limit a probiotic to feed supplements or to animals would likely be more applicable today. The scientific basis for the development of probiotics stems from the knowledge that the gut flora is involved in protecting the host animal against colonization of the intestinal tract by enteroinvasive or opportunistic nonindigenous organisms.

Although much of the knowledge that predates Metchnikoffian theories is not well established, belief in the health-promoting properties of lactic acid bacteria (LAB) was well ingrained in Eastern European and Russian traditions before Metchnikoff's publications (156, 159). Healthy animals and humans have normal microflora that are

present on internal and external surfaces. The skin, upper respiratory tract, lower urogenital tract, oral cavity, and gastrointestinal tract all represent microbial habitats, each of which has complex and different environmental conditions. In neonates, these microbial habitats receive microbes directly from the mother and also are contaminated during and directly following birth by the surrounding environment (15, 28, 143, 146).

Colonization of the body surfaces may be temporary or permanent, with mature microflora being dependent on the specific animal species, the individual animal, and the inhabiting environment (64, 146, 179). The well-established indigenous microflora of the body surfaces are quite stable. Through competitive exclusion (44, 162, 182) or colonization resistance (23, 24, 179), the colonization of the surfaces by nonindigenous microorganisms is inhibited (146, 162).

The importance of indigenous microflora received renewed interest when intestinal disturbances in mice were produced using oral antibiotics (179). Protective effects of normal gut microflora have been demonstrated for *Salmonella typhimurium* (97, 161), *Escherichia coli* (35,), *Campylobacter* (135, 162), and *Yersenia enterocolitica (163).* Several factors can influence the prevention of early colonization by microorganisms and the development of normal microflora. The lack of breast feeding is one factor that is thought to contribute to a deficiency of normal microflora development in infants (17, 28, 139).

The Use of Lactic Acid Bacteria as a Probiotic

There is considerable interest in understanding how the microflora of the intestines can be manipulated, particularly by dietary factors (30). Among food products

targeted as possible microflora manipulators, fermented dairy products have received considerable attention. LAB are an integral component of many dairy products. The term LAB refers to gram-positive, anaerobic microaerophilic or aero-tolerant, catalase-negative rods or cocci that produce lactic acid as an important product from the fermentation of sugars. Substantial evidence suggests that LAB have an inhibitory effect on pathogenic bacteria in the intestinal tract (18, 35, 49, 50, 51, 59, 62, 90, 103, 109, 117, 130, 185, 189). Several theories have been proposed to explain this bacterial antagonism. The most widely accepted theory is that of competitive exclusion, which holds that LAB control the growth and colonization of opportunistic enteric pathogens by effectively competing for nutrients, lowering the luminal pH through the production of lactic acid and the possible production of antibiotic-like substances. This theory is the basis for the use of LAB as a biological prophylaxis for enteric diseases (23, 162, 182). The long-standing belief that cultured dairy foods containing active LAB are beneficial in the prevention and treatment of enteric diseases was first proposed by Metchnikoff (107) in 1907, investigated further by Vincent et al. (178), and more recently studied by Wood (185).

Various explanations have been proposed for the beneficial effects demonstrated by cultured dairy foods. The involvement of LAB gangliosides, however, has not been considered. LAB such as members of the genera *Lactobacillus* and *Streptococcus* are used in the commercial production of fermented dairy products. Gangliosides constitute about 1% to 2% of the total lipid content of buttermilk (65). The possible involvement of LAB in the modification or production of milk-fat gangliosides also has been suggested by the identification of a unique ganglioside 9-0-acetyl-GD_3 in bovine buttermilk (14).

Goldin et al. (49, 50) conducted research on the oral administration of strains of *Lactobacillus acidophilus,* which decreased the concentrations of some fecal enzymes. The enzymes that are decreased (β-glucuronidase, azoreductase, and nitroreductase) have the ability to transform procarcinogens to carcinogens in the colon (58, 68). The beneficial effects suggested by these studies have encouraged further investigation by others concerning the effects of commercial fermented products containing lactic acid bacteria 100, 104).

The belief that breast feeding provides increased resistance to microbial infections, thereby lessening digestive problems, has been supported in several studies (17, 34, 50, 57, 69, 73, 111) and presents a good case for probiotic use. The neonatal tract is initially germ free, but it is progressively colonized with bacteria from the mother as well as external sources (15, 17, 34). The microflora of the gastric contents of a 5- to 10-minute-old infant were found to already reflect the cervical flora of its mother (15, 28).

It is still a mystery how the neonatal gut selects a limited number of microbial species from the enormous number to which it is exposed. The complex microflora that develop are largely responsible for protection against many infectious diseases and play a major role in resisting further colonization (23, 51). These protective microflora can be modified by various factors, such as diet (49, 139), environmental changes (185), and antibiotic treatment (130, 179), which result in a disturbance of the normal gut flora. This disturbance frequently enables pathogens to multiply and is a major cause of diarrhea. Infants ingesting milk-formula substitutes or milk from other species do not have the same degree of microbial resistance as breast-fed infants (17, 85). In breast-fed

newborns, colonization of the intestine by *Lactobacillus bifidus* occurs quickly. In non-breast-fed infants, other commensal microorganisms inhabit the intestine and become established (15, 34). The magnitude of lactobacilli in breast-fed infants can be attributed to the high concentration of a specific growth-promoting factor for *L. bifidus* present in human milk (69, 85). The high concentration of lactose in this growth promoter is believed to ensure that, even after the food has passed into the large intestine, there is sufficient lactose to produce acidic conditions due to lactose fermenters and to encourage lactobacilli growth (23, 49, 50). Specifically, *L. bifidus* helps convert lactose into lactic acid and acetic acid. The increased concentration of these acids in the intestinal tract lowers the pH and interferes with the growth of many enteropathogenic organisms (23, 49, 103).

The growth-promoting factor for *L. bifidus* is a nitrogen-containing polysaccharide with high lactose content, low protein, low bulk, and low buffering capacity (69). This growth promoter is found in bovine milk in only negligible amounts (69, 79, 85). In addition to the lactobacilli growth-promoting factor, other defense factors found in human milk include an anti-staphylococcal agent, immunoglobulins, complement components, lysozyme, peroxidase, lactoferrin, macrophages, lymphocytes, and lipids (57, 69).

Since Metchnikoff (104) conducted his studies at the turn of the century, the belief has persisted that LAB and cultured dairy foods produced by LAB hold the key to prolonged life and improved health. The ability to significantly increase the numbers of lactobacilli in feces within a few days by ingesting low-fat milk has been proven (18, 49, 189).

More recently, researchers have suggested that the use of fermented milk could have therapeutic effects on malnourished and diarrheal patients(2, 49, 116, 150, 156). Examples are the resistance to *Shigella sonnei,* which was enhanced by feeding fermented milk for 8 days (117), and the increased resistance to *Salmonella typhimurium* in subjects fed fermented milk (97, 130). About 40% to 70% of children with *Salmonella* and *Shigella* dysentery recovered when acidophilus milk was administered for a short time. Continued long-term administration of acidophilus milk resulted in 100% recovery (97, 130).

Lactic acid bacteria therapy also has been reported to have positive effects in the prevention and treatment of travelers' diarrhea (103, 106, 156). This condition is normally attributed to enterotoxigenic strains of *Escherichia coli (E. coli).* These *E. coli* cause diarrhea through their ability to colonize the small intestine and produce enterotoxin (64, 119, 127, 143). The mechanisms by which LAB may prevent or lessen the effects of diarrhea (e.g., the production of anti-*E. coli* [2, 27, 109], the synthesis of lactate with ultimate reduction in intestinal pH [23, 49], detoxification of endotoxin [130], prevention of toxic amine synthesis [154], and adhesion to the gut wall preventing colonization [2, 18, 130]) have been suggested in a number of reports. Treatment of bacterial infections by competitive inhibition of bacterium-receptor interactions has been proposed and successfully demonstrated (97, 103, 125, 130, 139, 150).

Adhesion of *E. coli*

Enteric diseases, including enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and enterotoxigenic *E. coli* (ETEC), all cause diarrhea, with ETEC being the most common cause of diarrhea in young farm animals (64). Attachment of enterotoxigenic bacteria to the susceptible host tissue is the initial step in the disease process (119, 159). Most strains of *E. coli* are normal inhabitants of the gastrointestinal tract. However, the expression of virulence factors, or mechanisms of action, that enhance an organism's ability to cause a variety of intestinal infections and neonatal diarrhea in animals and humans has been well documented (5, 31, 34, 39, 40, 111, 119). Pathogenic bacteria can cause diarrhea by (a) producing toxins that increase secretion by the crypt cells of the small intestine; (b) invading the intestinal mucosa, causing inflammatory reactions, which in turn increase secretion by crypt cells; or (c) destroying absorptive epithelial cells, which causes malabsorption (119, 143, 159).

Two important factors that provide ETEC with means of colonization are the expression of pili, which allow for the attachment of bacteria to the intestinal wall, and the production of enterotoxins that influence intestinal secretions (39). Pili are finger-like projections protruding from the bacterial surface; they are also designated as fimbriae, surface adhesions, and colonization-factor antigens. Pili enable bacteria to adhere to the small intestinal epithelium (19, 39, 47, 56, 123). The pili are filamentous proteins that are located on the surface of the bacteria. Normally, peristalsis and the flow of intestinal contents, coupled with the continuous surge of mucous over the surface of the constantly moving intestinal villi, keep noncommensal bacterial populations small (5, 119, 143).

The ability of bacteria to adhere to the mucosal surface via pili would improve the capacity for intestinal colonization by the organism.

Different types of pili are synthesized by various strains of *E. coli,* which allow the bacteria to bind to the intestinal tract of several species. Antigens found on *E. coli* that cause diarrhea in pigs are K88, K99, F41, and 987P; K99 and F41 antigens cause diarrhea in calves; K99 antigens are present on *E. coli* that cause diarrhea in lambs; and the colonization-factor antigens, CFA I, CFA II, and CFA IV, are partially responsible for diarrhea in humans (64, 110, 119). In general, one *E. coli* strain expresses one type of antigen (64, 119), although there are some exceptions (149). Both glycoprotein (160, 165) and glycolipid (171) moieties have been implicated as receptors for K88 fimbriae in the small intestines of pigs.

The mucosa of the intestine is the site of both absorption and secretion. The crypt portion contains undifferentiated epithelial cells, which are the major sites for secretions (5). These cells later proliferate and migrate out to the villi, thus losing their proliferative and some secretory abilities. Crypt cells then become the sites of absorption and digestion in the small intestine (111, 119). Absorption occurs more than secretion in normal intestinal function. Intestinal secretion is regulated by cyclic adenosine monophosphate (cAMP), which is in turn regulated by specific hormones (111, 119, 143). Enterotoxin produced by *E. coli* is believed to act similarly to hormones, signaling the increase of mucosal cAMP. The increase in cAMP causes the hypersecretion of the crypt cells; this leads to increased secretion and reduced absorption, resulting in diarrhea (119).

The specificity of bacterial pili and the presence of receptors for these pili producing clinical manifestations have been best evidenced for *E. coli* bacteria possessing

the K88 fimbrial adhesion, which causes diarrhea in neonatal pigs (36, 72, 149). The presence of two plasmids, one encoding the genes for the expression of K88 fimbriae and the other encoding the genes causing toxin production, has been found to correlate directly with the incidence of diarrhea in neonatal pigs (146, 149). It has been further demonstrated that the adhesion of bacteria to the intestinal brush borders is facilitated by the K88 pili (36). Possession of the receptor for K88 pili has been proven to confer sensitivity, whereas the lack of this specific receptor has been discovered in pigs that are resistant to the K88-mediated diarrhea (12, 153, 160). In addition, K88-specific receptors have been identified in the mucous layer that covers epithelial cells of the small intestines of neonatal pigs (11, 12). The K99 pili possess an affinity for neuraminic acid derivatives (84). Recent research has indicated that a receptor-active glycolipid (monosialoganglioside II^3 NeoGc-Lac) is present in the intestines of pigs (124). This same receptor also has been identified as a receptor in horse erythrocytes (158).

Hemagglutination

The binding or adhesive properties of *E. coli* were first examined by Guyot (56), who found that some strains had the ability to agglutinate erythrocytes (56). In 1955, Duguid (31) was the first to link the attachment property of *E. coli* (agglutination) to fimbrial projections on the surface. Hemagglutination has proven to be an important way to detect and classify pili, as well as to indicate their presence. Distinction of pili as mannose-sensitive (MS), which are inhibited by mannose and designated as Type I pili, or mannose-resistant (MR), which are not affected by mannose and designated as Type II pili, represents one form of classification (40, 126). ETEC possesses mannose-resistant

pili. The K88 antigen will hemagglutinate guinea pig and chicken erythrocytes (31, 32). The 987P antigen exhibits only weak MR-hemagglutination with chicken erythrocytes (39). The K99 antigen shows MR-hemagglutination with horse and sheep erythrocytes (94, 158). The F41 antigen demonstrates MR-hemagglutination with sheep erythrocytes (47, 92). The CFA I antigens possess MR-hemagglutination with human, bovine, and chicken erythrocytes, whereas the CFA II antigen has MR-hemagglutination with chicken and bovine erythrocytes (38, 39, 42).

The relationship between hemagglutination and adhesion was first established by Evans et al. (37). Observations indicated that strains of *E. coli* from the intestines and urinary tracts of diseased individuals showed increased hemagglutination with a variety of cell types, as compared to normal intestinal microflora. This association has been established for MS-hemagglutination of guinea pig erythrocytes with a variety of cell types (123). Determining the specific membrane receptor molecules on erythrocytes and epithelial cells is the most important factor in establishing the relationship between hemagglutination and adhesion.

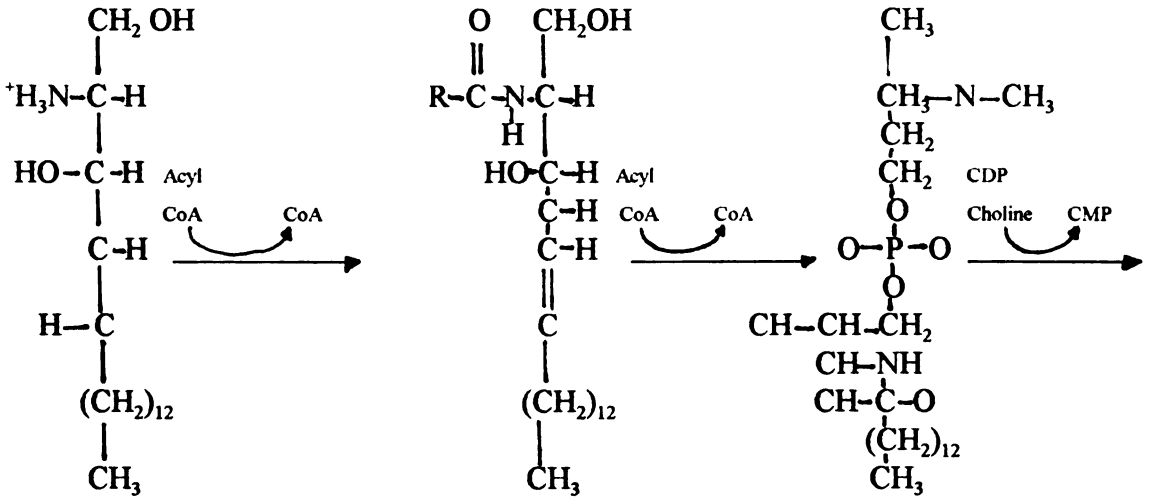
High concentrations of N-acetyl neuraminic acid have been found to inhibit hemagglutination. Lindahl et al. (93) demonstrated that disialogangliosides (Type II, Sigma) and highly purified GM2 gangliosides caused specific inhibition of hemagglutination and binding of strains to isolated human intestinal epithelial cells. In addition, researchers have found that terminal sialic acid residues inhibited $K99^+$ *E. coli* and CFA II *E. coli* (93, 94, 99, 116, 120, 183).

Gangliosides

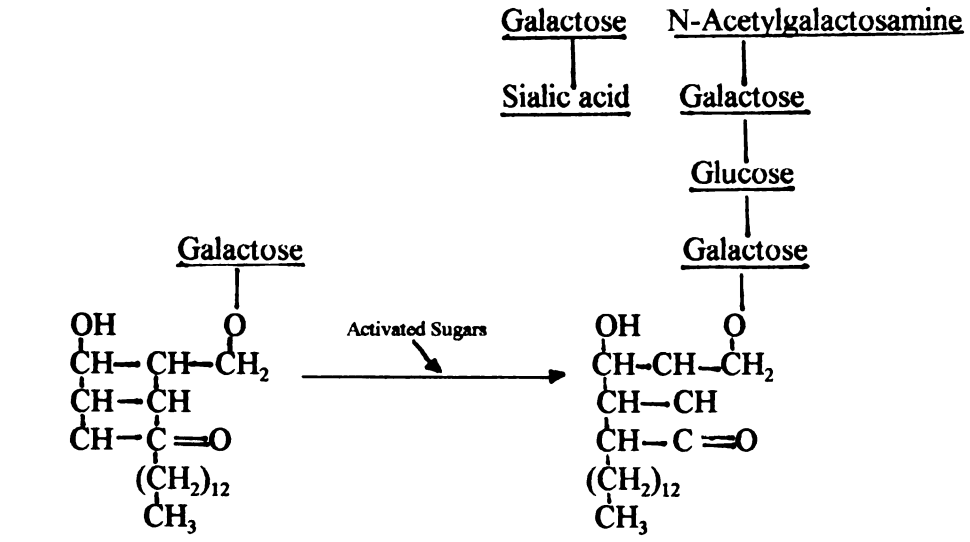
Glycolipids consist of carbohydrate and lipid moieties in covalent linkage. Two important classes are cerebrosides and gangliosides, with the term "glycosphingolipid" being a more accurate description of both groups. Gangliosides are naturally occurring derivatives of cerebrosides containing a more complex carbohydrate moiety that includes one or more N-acetyl neuraminic acid, also called sialic acid, units in the oligosaccharide chain that is attached to ceramide (75, 88). (Figure I.1.)

Gangliosides are normal components of the plasma membrane and are abundant in the nervous system. The ganglioside's hydrophilic oligosaccharide chain is oriented toward the extracellular environment, protruding on the membrane surface (88, 173). The ganglioside pattern is well defined, but it varies greatly between the same tissues of different species and also between species (75, 76). Sialic acid is the general term for the series of compounds derived from neuraminic acid (5-amino-3, 5-dideoxy-D-glycero-galactononulosonic acid). The major types of sialic acid detected in gangliosides are N-acetylneuraminic acid and N-glycolylneuraminic acid (88, 173). The other carbohydrates present in gangliosides are glucose, galactose, and either N-acetylgalactosamine or N-acetylglucosamine (88).

The biosynthesis of gangliosides (Figure I.2) occurs in the Golgi apparatus and involves the sequential addition of saccharide units to ceramide, with each reaction owing its catalyzation to glycosyltransferase. The strict specificity of many glycosyltransferases holds the key to the formation of different gangliosides (75, 148, 164). Lactosyl-ceramide is the precursor for all the gangliosides of the ganglio-series. Early insertion of sialic acid into lactosylceramide leads to the ganglioside chain carrying a single sialic acid



SPHINGOSINE **CERAMIDE** **SPHINGOMYELIN**



GALACTOCEREBROSIDE **GM_1 GANGLIOSIDE**

Figure I.1: The structures of important ganglioside precursors.

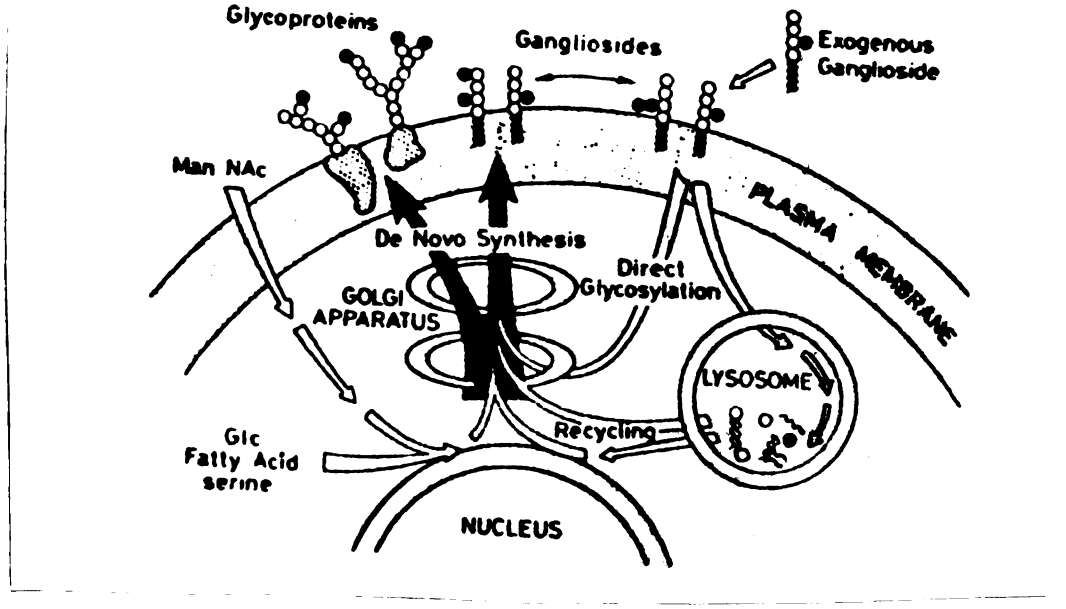


Figure I.2: Schematic drawing of ganglioside biosynthesis. From G. Tettamanti et al., 1988.

residue linked to an inner galactose at the 3 position. The designation of ganglioside monosialic acid 3 (GM3) is given to this specific ganglioside. Designations of GD3 and GT3 are given to ganglioside disialic acid 3 and ganglioside trisialic acid 3, respectively.

An important factor leading to the testing of glycolipids as receptors for bacterial adherence is the amount of diversity within this group (7, 75, 88, 121). Carbohydrate molecules of varying complexities are normal features of cell surfaces throughout nature and are termed collectively the glycocalyx (7). The glycocalyx encompasses glycoproteins and glycolipids (which include gangliosides), as well as the mucosal coating on epithelial surfaces, including the intestinal tract. These carbohydrate moieties are usually in the form of oligosaccharide chains. The sugar sequences comprising these chains show a high degree of complexity and variability, genetically determined, which provides them with broad variability in their biological functions. These functions include immunological specificity, cell-to-cell adhesions, and receptor-site capabilities (75, 76, 87, 113, 115, 164). This diversity increases the possibility of these compounds being specific receptors in many systems (164).

Binding has yet to be attempted using specific gangliosides as competitive inhibitors of bacterial pili. Since the intestinal receptor sites for bacterial pili have been demonstrated to be glycolipid in nature, the attempt to use gangliosides as a competitive binding site is a relatively new approach (21, 44, 46, 54, 99, 150).

Cell-surface gangliosides have been shown to function as receptors for a variety of toxins, lymphocytes, and biological mediators (4, 164). Gangliosides have also been associated with tumors and used as differentiation markers (20, 22, 48, 61, 66). There is substantial evidence to support the role of gangliosides in the regulation of immune

functions. The documented immune-regulative properties of gangliosides include (a) inhibition of normal human lymphocytes' response to mitogens and antigens (52, 86, 118, 132); (b) inhibition of cell proliferation, which is interleukin-2 dependent (105); (c) inhibition of cytotoxic functions (118, 169); and (d) binding of CD4 on the T-lymphocytes (78, 113, 122). These applications appear to have relevance in the area of active immunotherapy with vaccines (96, 114, 115, 168).

Antimicrobial Properties of Gangliosides

Research has indicated that substances found in milk, especially colostrum, are effective in immunologically protecting young animals as well as humans from diseases (17, 50, 51, 57, 69, 125, 150). Milk, however, contains factors other than immunoglobulins that also possess antimicrobial properties. Non-immunoglobulin fractions identified in milk that have bacteriostatic or bacteriocidal effects include chemotactic factors, lacto-ferrin, lysozyme, lactoperoxidase, interferon, and bifidous factors (2, 73, 109).

Recently, lipid components of milk have been under investigation due to the discovery of antimicrobial activity in this fraction. The antimicrobial activity resisted boiling and digestion with trypsin but could be rendered ineffective by periodate treatment (2, 73). This demonstrates that the activity is likely related to carbohydrate residues, which may act as cell-surface receptors. Gangliosides naturally occur in milk fat, as part of the milk fat globular membrane, and exhibit antimicrobial and enterotoxin-inhibiting capabilities (2, 63, 79, 85, 87, 99).

Studies have shown that cell-surface gangliosides serve as cell-recognition sites for the attachment of viruses, bacteria, and bacterial toxins (2, 13, 44, 54, 55, 76, 77, 78). This attachment is attributed to the sialic acids and oligosialyl structures in glycolipids, which are associated with the microorganism's cell wall (5, 146). Researchers now are suggesting that the binding ability of gangliosides influences the growth, infectivity, and pathogenicity of some microorganisms (44, 52, 60, 61, 68, 80, 82, 86). Lee and his colleagues (89) found indirect evidence that milk oligosaccharides can bind to *Pseudomonas aeruginosa* and inhibit the attaching of this organism to mucous. It has also been found that the exotoxin of *Vibrio cholerae* can be neutralized by the binding of gangliosides (63, 177) and that the binding of heat-liable enterotoxins from porcine and human enterotoxigenic *E. coli* can be inhibited by gangliosides (150).

Antibacterial Treatment of Gastric Ulcers

The acidic pH of the human stomach has caused scientists to conclude that bacterial growth is not a problem in the stomach. In 1983, Marshall and Warren (101) cultured a bacterium from a human gastric mucosa and showed an association between its presence and gastric inflammation. This organism was named *Helicobacter pylori (H. pylori);* it infects populations worldwide (9, 10). There is a strong association between infection with *H. pylori* and gastric ulcers, with 70% of gastric ulcer patients testing positive for *H. pylori* infection (131, 135, 175). If patients suffering from ulcers induced by non-steroidal anti-inflammatory drugs are excluded, the association between *H. pylori* and gastric ulcers increases to 96% (9, 10, 53, 131). Superficial gastritis can result from the ingestion of *H. pylori.* If *H. pylori* is removed from the system, the

gastritis is alleviated (9, 53, 131). *H. pylori* is found almost exclusively in the stomach, where it is closely associated with gastric epithelial cells or the mucosal layer. Critical to the bacteria's survival in the stomach is the production of urease. The production and subsequent release of urease facilitate the production of ammonia, which functions as a base and raises the pH of the immediate environment. *H. pylori* can therefore survive well in the presence of urea (102).

Adhesion is an important prerequisite for many bacteria that colonize the mucosal surfaces and may be important for the induction of inflammation by *H. pylori* (9, 74). *H. pylori* expresses sialic-acid-specific hemagglutinins and also lipid-binding adhesions (95, 157). The *H. pylori* hemagglutinin also has been shown to have a high affinity for the N-acetylneuraminyl-lactose (2-3 linkage) that predominates in bovine colostrum (95, 108, 157).

CHAPTER II

GANGLIOSIDE EXTRACTION AND PURIFICATION FROM LACTIC ACID BACTERIA, CULTURED DAIRY FOODS, AND BOVINE MILK FAT

Abstract

Gangliosides are a diverse group of naturally occurring complex sugar-containing glycosphingolipids, which have sialic acid (N-acetylneuraminic acid) as an essential component, as well as a hydrophobic, lipid component (ceramide). Brain, visceral organs (especially the liver), erythrocytes, and other tissues contain a large number of different gangliosides (75, 121, 148). Gangliosides of different types naturally occur in the milk fat of all mammalian species, as part of the milk fat globular membrane, which is derived from the plasma membrane of epithelial cells in the mammary gland (79, 170). The average concentrations of gangliosides in the cow mammary gland and milk are 88.4 and 5.6 nmol/g wt, respectively, with nearly 90% of the milk gangliosides contained in the milk fat globular membrane (79). Although the literature on this subject is incomplete, it seems that the ganglioside compositions of milk fat from different mammalian species can vary markedly (79, 85).

The ability to extract large amounts of crude mixed gangliosides from powdered buttermilk and yogurt preparations was essential to obtaining purified ganglioside samples for further study. Several procedures were examined. The initial protocol

(Protocol 1) was based on Folch-like extraction as modified by Svennerholm and Friedman (168). Sweet and cultured buttermilk were extracted at Oakland University, while simultaneous extractions were performed on lactic acid bacteria (LAB) at Michigan State University (MSU) laboratories. Identical procedures were used for extractions at both laboratories. Subsequent attempts to remove non-ganglioside contaminants were performed at Dr. Kathleen Moore's laboratory at Oakland University and led to the use of other protocols. Protocol 2 used an acetone pre-extraction step to remove natural lipids; the extracted buttermilk powder was then processed as in Protocol 1. In Protocol 3, buttermilk powder was resuspended in water, dialyzed, and lyophilized before extraction by means of Protocol 1. Protocol 4 also involved pre-extraction dialysis and lyophilization; the resuspended powder was then extracted with a series of chloroform/methanol solutions and subsequently partitioned (138). Ultimately, an extraction and Diethylaminoethyl (DEAE)-Sephadex A-25 (Sigma Chemical) chromatography procedure as described by Bonafede et al. (14) yielded what were termed purified preparations. These purified mixed ganglioside preparations, when quantitated by the resorcinol assay, produced yields significantly lower than the crude preparations. The yields from purified preparations produced a true purple/blue color (indicative of gangliosides when sprayed with resorcinol) as opposed to the murky purple/brown color obtained with crude preparations. The subsequent mixed ganglioside extractions were all performed utilizing the purified preparation technique.

Introduction

There is substantial evidence that LAB have an inhibitory effect on pathogenic bacteria in the intestinal tract (94, 103, 109, 178). Several theories have been proposed for this bacterial antagonism; the most widely accepted theory is that of competitive exclusion. This theory holds that LAB control the growth and colonization of opportunistic enteric pathogens by effectively competing for nutrients, lowering the luminal pH by producing lactic acid, and possibly producing antibiotic-like substances. When an imbalance occurs in this arrangement, pathogens gain an advantage, ultimately resulting in a disease state. This rationale is the basis for the controversial use of LAB as a biological prophylaxis for enteric diseases in humans (45, 103).

Since the initial hypothesis of Metchnikoff (107), it has been believed that cultured dairy foods containing LAB are beneficial in the prevention and treatment of enteric diseases. LAB used for manufacturing cultured dairy foods have been shown to modify the metabolic activities of the colonic microflora (103), possess antimicrobial activity (178), and neutralize *E. coli* enterotoxin (109). Although various mechanisms have been proposed for these beneficial effects, the involvement of LAB gangliosides has not been considered as a mechanism.

Sialic acids have been detected in the cell membrane of *Lactobacillus* and *Streptococcus spp.* (4, 144). Also, 1% to 2% of the total lipid content of buttermilk is ganglioside, consisting of neuraminosyl lactosyl ceramide (20%) and dineuraminosyl lactosyl ceramide (50%) (65). An unusual ganglioside, 9-0-acetyl-GD_3, has been found in bovine buttermilk (14), which suggests a possible involvement of LAB in modifying milk fat gangliosides or producing other gangliosides not present in the original milk fat.

Mixed gangliosides were extracted from cultured dairy foods (cultured buttermilk, yogurt), sweet buttermilk, LAB, and evaporated milk using a modification of the method of Bonafede et al. (14). The extraction process, which was labor intensive and had not previously been developed specifically for cultured dairy foods (CDF), bovine milk fat (BMF), or LAB, required the application of existing protocols to the unique and specialized challenges of ganglioside extractions from these products. In addition to the extraction process, mixed gangliosides were purified with a DEAE- Sephadex A-25 (Sigma Chemical) column, producing samples with fewer contaminants. Although LAB ganglioside extraction initially was undertaken at MSU, the major task of ganglioside extraction from dairy products (cultured buttermilk, sweet buttermilk, yogurt, evaporated milk) and LAB occurred at Dr. Kathleen Moore's laboratory in the Department of Chemistry at Oakland University.

Materials and Methods

Ganglioside Sources

Two starter cultures were used. One (designated 8803) was supplied by Darigold, Seattle, WA, and used in their cultured buttermilk product. The second (designated R-39) was supplied by Chris Hansen's laboratory, Milwaukee, WI, and used in producing the yogurt supplied by the MSU Dairy Plant. The starter cultures contained a mix of different bacterial strains, including *Streptococcus lactis, Streptococcus lactis* subspecies *diacetylactis, Streptococcus cremoris,* and *Lactobacillus citrovorum.* The specific amounts of each bacteria strain are a trade secret and therefore, are not available for reporting.

Cultured buttermilk was prepared from skim milk treated with the starter culture, and sweet buttermilk was derived from the liquid remaining after butter was processed. Both buttermilks were obtained in the powdered form. Yogurt is also produced with a starter culture, usually containing *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Mixed ganglioside extractions and purifications were performed on LAB obtained from a buttermilk starter culture (Darigold, Seattle, WA), sweet and cultured powdered buttermilk (Darigold, Seattle, WA), and plain cultured yogurt (MSU Dairy Plant, East Lansing, MI) using a modification of the method of Bonafede et al. (14).

The difficulty in growing large batches of LAB in order to extract gangliosides proved to be a major challenge. Various media were used to facilitate growth of LAB from a starter culture. Media tested included de Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories, Detroit MI) , 1/2 MRS with 1% lactose (Sigma Chemical), MacConkey's medium (Difco), and a powered milk-based medium. The most successful growth occurred using the 1/2 MRS broth fortified with 1% lactose. Because of the small yields of gangliosides produced through this labor-intensive method, the previously described starter cultures were ultimately used as ganglioside sources.

Initial Ganglioside Extraction Procedures

The initial extractions were based on a modification of the method of Svennerholm and Friedman (168). This procedure depends on the ability of gangliosides to partition from the chloroform-methanol phase into which they are extracted, into an upper aqueous phase. This partitioning should ensure that most of the other undesired lipids remain in the chloroform phase.

One hundred grams of dried dairy product were dissolved in 318 ml of deionized water. This solution was stirred for 8 min using a magnetic stirrer bar. Then 455 ml of methanol (MeOH) and 228 ml of chloroform ($CHCl_3$) were added, and stirring continued for 30 min. The solution was poured into a separatory funnel and allowed to stand for 4 h until two distinct phases were observed. The upper phase was set aside for later use. To the lower phase, MeOH at 1/2 the remaining volume (approximately 250 ml) and 0.01 M KCl in water at 1/3 the volume of the lower phase (approximately 173 ml) were added and left to stand overnight. The upper phase was then combined with the previous upper phase, rotary evaporated, and ammonium sulfate added until saturated (approximately 60 to 70 g), and stirred 15 min. The sample was centrifuged for 20 min at 10,000 rpm and the liquid poured into dialysis tubing. The sample was dialyzed against running tap water for 4 to 6 h and refrigerated at 4°C overnight. The tap water was replaced with deionized water at 4°C for 24 h, the water changed, and kept at 4°C an additional 24 h. The dilute preparation was removed and lyophilized to produce a concentrated crude preparation. The lyophilized sample was resuspended in deionized water and used for thin-layer chromatographic (TLC) analyses. These preparations were subjected to TLC analyses; sialic acid standards and ganglioside standards (purified mixed brain gangliosides and Disialoganglioside 3 (GD_3) were routinely run with samples.

In an attempt to obtain better yields and cleaner preparations, four different protocols were employed in the isolation of crude ganglioside mixes. Protocol 1 involved a Folch-like extraction as modified by Svennerholm and Friedman (168); this procedure was described above. Protocol 2 used an acetone pre-extraction step to remove neutral lipids (170); the extracted buttermilk powder was then processed as in Protocol 1. In

Protocol 3, the buttermilk powder was resuspended in water and dialyzed and lyophilized before extraction by means of Protocol 1. The dialysis step has been shown to remove more than 50% of the powder solids (138). Protocol 4 also involved pre-extraction dialysis and lyophilization; the resuspended powder was then extracted with a series of chloroform/methanol solutions and partitioned as described by Ren et al. (138).

Purified Ganglioside Extraction Procedure

Cultured buttermilk was prepared from skim milk treated with a starter culture (designated 8803 and supplied by Darigold, Seattle, WA) , and sweet buttermilk was derived from the liquid remaining after butter was processed. Both buttermilks were obtained in the powdered form. Yogurt is also produced with a starter culture, (designated R-39 and supplied by Chris Hansen's laboratory, Milwaukee, WI.). The yogurt starter culture usually containing *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Mixed ganglioside extractions and purifications were performed on LAB obtained from a buttermilk starter culture (Darigold, Seattle, WA), sweet and cultured powdered buttermilk (Darigold, Seattle, WA), and plain cultured yogurt (MSU Dairy Plant, East Lansing, MI) using a modification of the method of Bonafede et al. (14).

Specifically, 100 g of dried dairy product (powdered sweet buttermilk, powdered cultured buttermilk, or lyophilized yogurt) were suspended in 1,000 ml of chloroform: methanol (1:1) and allowed to stir overnight. After solid material was removed by Buchner filtration, the extract was subjected to rotary evaporation for removal of solvents. The dried extract was resuspended in 50 ml of water and dialyzed against deionized water for 2 days in a cold box. The dialyzed sample was lyophilized and

resuspended in chloroform:methanol:water (C:M:W; 30:60:8) for anion-exchange chromatography. The sample was applied to a 1.5 cm x 30 cm column of DEAE-Sephadex A-25 (Sigma Chemical), which had been preequilibrated with the C:M:W solvent. The column was then washed with 175 ml of the C:M:W solvent and 75 ml of methanol. Gangliosides were eluded from the column with 150 ml of 0.4 M ammonium acetate in methanol. Ganglioside-containing fractions were pooled, and methanol was removed by rotary evaporation. The dried ganglioside fraction was resuspended in deionized water and dialyzed against deionized water for 2 days to remove ammonium acetate. The dialyzed ganglioside fraction was lyophilized and resuspended in minimal deionized water for quantitation, TLC analyses, and hemagglutination studies. The ganglioside fractions were stored at -32^{0}C and thawed to room temperature prior to use.

Lactic Acid Bacteria Ganglioside Extraction

LAB gangliosides were extracted in a manner similar to purified gangliosides except that the LAB cells were ruptured by glass beads in a chilled BeadBeator chamber (Fisher Scientific, Atlanta, GA) and cell membranes were recovered after centrifugation of homogenate. The membrane pellet was then extracted with a 20-fold volume of chloroform:methanol (1:1) and processed as described above.

Starter cultures used for the production of yogurt were obtained from Chris Hansen's laboratory (Milwaukee, WI). The starter culture (R-39) contained multiple strains of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. To produce large quantities of LAB for the isolation of the bacterial-cell-wall gangliosides, 0.2 ml of starter culture was inoculated per liter of MRS Broth (BBL, Becton Dickinson Microbiology

Systems, Cockeysville, MD) and incubated at 39.5 °C for 72 h. The resultant culture yielded approximately 7 g of packed wet cells per liter of medium when harvested by centrifugation at 7,650 x g for 20 min. Approximately 1,400 g of wet bacterial cells were initially propagated and harvested for ganglioside isolation using this method.

The starter culture (8803) for buttermilk, which was obtained from Darigold, Seattle, WA, was also cultured. The bacterial strains used in this starter culture were proprietary; therefore, the specific bacteria strains used were not identified. The bacterial-cell-wall gangliosides were isolated and purified at Oakland University in the laboratory of Dr. Kathleen Moore.

With respect to the isolation of gangliosides from LAB, the typical preparation involved motor-driven homogenization of 60 g of bacterial cells (wet weight) with 100 ml of sucrose phosphate buffer, pH 7.4. The mixture was then transferred into a chilled BeadBeator chamber (Fisher Scientific, Atlanta, GA). The bacteria cells were ruptured by glass beads for three 1-min intervals; each 1-min process was followed by a 10-min cool-down. The process was done in a cold box.

After treatment was complete, the beads were allowed to settle before decanting of the homogenate. The beads were washed twice with 50 ml of buffer; the washes were then combined with the homogenate. The homogenized bacteria mix was centrifuged for 20 min at revolutions per minute = 25,000. This procedure pelleted the bacterial membranes, which contained most of the gangliosides. The supernatant was lyophilized and then extracted for its minimal ganglioside content. The bacterial-membrane pellets were weighed and gradually homogenized with chloroform:methanol (1:1 v/v, 20 ml of solvent per gram of pellet). After overnight extraction, the process continued as

described earlier, according to the method of Bonafede et al. (14). Eventually, lyophilized samples were extracted by Dr. Moore's laboratory using the method previously described.

Ganglioside Quantitation

Thin layer chromatography (TLC) was performed using silica gel 60 TLC precoated plates (glass plates 10 x 10cm Merck). The plates were activated at 100° C and ganglioside preparations and ganglioside and N-acetylneuraminic acid (NANA) standards were spotted on silica gel G plates and placed in a chamber containing chloroform: methanol:0.25% $CaCL_2$ solution (30:18:4). Plates were developed in the chambers for twice the time it took the solvent to reach the top of the plate. Plates were then removed, dried, sprayed with resorcinol-HCL for visualization, and heated at 110°C for 15 min. Sialic-acid-containing material gave blue spots; organic material lacking sialic acid charred brown. Densitometry of TLC plates was performed with a Shimadzu Dual-Wavelength TLC-Scanner CS930. The chromatograms were obtained by single wavelength zig-zag scanning of each lane at 620 nm (beam dimension: 0.4 X 0.4mm) in the reflection mode (168). The purified preparations yielded blue bands of varying patterns. All types of preparations had a band that migrated with standard GD_3; LAB preparations had only one additional band, whereas the dairy products had six to eight separate ganglioside bands. The yields resulting from this process were small, but the preparations were highly purified and devoid of extraneous contamination.

Results

Two basic methods were used to characterize the crude ganglioside mixes. Rough quantitation was obtained by the resorcinol/HCl spectrophotometric assay method (168). This assay quantitates sialic acid content of the preparations, but larger quantities of ganglioside may be artifactually suggested due to enhancing interference by other partially reactive contaminants in our crude preparations. Appendix A.1 (Appendix p. 33-35) contains an inventory of crude mixed ganglioside preparations by protocol (1 through 4); included are the volume extracted, the ganglioside concentration, and the total ganglioside present on a micromole (μmol) basis. The quantitative assessment of our buttermilk and yogurt preparations are summarized in A.2 (Appendix p. 36).

A summary of the present research with crude preparations is given in A.3 (Appendix p. 36). Aliquots of purified gangliosides were reacted with resorcinol-HCL, and absorption at 620 nm was measured. Sample absorption was compared to that of NANA standards to determine sialic acid content. Mean yields on a nmol/g dry weight basis were as follows: sweet buttermilk = 11.6 ± 8.1, cultured buttermilk = 7.3 ± 5.0, yogurt = 13.2 ± 5.6, yogurt starter culture LAB mix (Chris Hansen R-39) = 16.7 ± 1.5, and buttermilk starter LAB mix (Darigold 8803) = 65.0 ± 13.7. The crude mixed ganglioside preparations were evaluated qualitatively by examining thin-layer chromatograms. The developed plates were sprayed with resorcinol/HCl reagent and heated; sialic-acid-containing bands stained a distinctive purple/blue color, whereas non-sialic-acid contaminants gave nondistinctive brown spots. The brown material was frequently at the origin and occasionally migrated into the plates. In terms of qualitative observation of TLC plates in the bacterial preparations, a band that correlates with disialoganglioside-3 was predominant, with a fainter, higher mobility unknown band. In

the yogurt preparations, up to eight bands, including disialoganglioside-3, were visible; in terms of color, the bands were moderate to strong in intensity. For the cultured buttermilk preparations, up to six bands, including disialoganglioside-3, were visible; again, color intensity was moderate. With the preparations from sweet buttermilk, up to six bands, including disialoganglioside-3, were visible; color intensity was moderate to strong. The TLC observations for the different protocols are summarized in A.4 (Appendix p. 37).

Protocol 4 gave fairly consistent quantitative yields, as well as good quality ganglioside bands. In addition, the mean value for sweet buttermilk (36.5 ± 13.9 μmol/ 100 g powder) was close to the expected yield from sweet buttermilk powder (48 μmol/ 100 g powder) (65). From comparison with standards, the primary component of crude ganglioside mixes from both the sweet and cultured buttermilk appears to be GD_3.

The average total sialic acid present in μmol for different dairy products and LAB from purified preparations A.5 (Appendix p. 38) was (a) cultured buttermilk = 1.4 μmol ± 3.3, (b) sweet buttermilk = 2.6 μmol ± 6.1, (c) yogurt = 2.18 μmol ± 3.25, and (d) LAB = 0.426 μmol ± 0.44.

Yogurt from the MSU Dairy Plant was subjected to extraction by means of Protocols 1 and 4. Briefly, Protocol 1 was based on Folch-like extraction as modified by Svennerholm and Friedman (168). Protocol 2 used an acetone preextraction step; the extracted buttermilk powder was then processed as in Protocol 1. In Protocol 3, the buttermilk powder was resuspended in water, dialyzed, and lyophilized before extraction by means of Protocol 1. Protocol 4 also involved preextraction dialysis and lyophilization; the resuspended powder was then extracted with a series of chloroform/

methanol solutions and subsequently partitioned (138). Protocol 1 extractions resulted in extremely high resorcinol quantitation values; these same preparations showed no evidence of ganglioside bands after TLC. Because of these unsatisfactory results, we switched to Protocol 4. Preparations performed using this protocol provided purer quantitation results, and TLC analysis usually resulted in an N-acetylneuraminic acid (NANA) band and some ganglioside bands (not GD_3). The preextraction dialysis was helpful in removing much of the unwanted yogurt solids; however, these preparations remained viscous.

Of the four protocols performed, Protocol 4 provided the best results in terms of purity, but still contained contaminants. Ultimately, an extraction and DEAE-Sephadex A-25 (Sigma Chemical) chromatography procedure as described by Bonafede et al. (14) yielded what were termed purified preparations. The purified preparations provided a low-yield, highly purified sample. The subsequent mixed ganglioside extractions were all performed using the purified preparation technique.

Discussion

The isolation of large amounts of crude mixed gangliosides from powdered buttermilk, yogurt, and LAB used to produce cultured dairy products was the initial goal of this part of the present research project, which was to be accomplished by means of a Folch-like extraction (168). This procedure (Protocol 1) was used for the LAB extractions at MSU and for the sweet and cultured buttermilk and yogurt extractions at the Oakland University laboratory. The difficulty in growing large batches of LAB in

order to extract gangliosides proved to be a major challenge. The most successful growth occurred using a 1/2 minimal MRS medium fortified with 1% lactose.

In general, crude preparations are high-yield, low-purity processes that are useful for estimating ganglioside content in dairy products and bacteria. Several different procedures were tried, resulting in a range of values. Purified preparations are high-purity, low-yield processes that are useful for *in vitro* anti-microbial studies. To eliminate interference by nonganglioside product components, a large percentage of gangliosides are lost in the process. The results of the purified preparation process are listed in A.6 (Appendix p. 38-39). The purified preparation procedures achieved the goal of decreasing the amount of contaminants present in samples, indicated by TLC results and a decrease in mixed gangliosides (actually a reduction in ganglioside contaminants). The lower yield of crude gangliosides from cultured buttermilk extracted by Protocol 4, Table A (Appendix p. 34-36) is reasonable because skim milk contains less than 8% of the normal milk gangliosides (65). The ganglioside content of our cultured buttermilk powder was probably derived primarily from the bacterial components of the cultured dairy product, due to the lack of fat content in skim milk. The dairy products, whether cultured or not, appeared to have a more diverse group of ganglioside components than did the LAB. When the purified mixed ganglioside preparations were quantitated by the resorcinol assay, the yields were significantly lower than those calculated for the crude preparations (sweet buttermilk = 1.62 ± 0.34 μmol/100 g powder; cultured buttermilk = 0.82 ± 0.89 μmol/100 g powder), but the color reaction was a true purple/blue, not a murky mauve. Likewise, TLC analysis showed smaller but purer amounts; therefore, it was decided to process all of our mixed ganglioside preparations by this method.

A.1: Inventory of ganglioside preparations, by various protocols.

Preparation No.	Volume (ml)	Concentration (mM)[a]	Total Gangliosides Present (μ)
PROTOCOL 1: CULTURED BUTTERMILK			
4	6.1	11.10	67.71
5	24.0	4.75	114.00
6	10.5	2.90	30.45
7	14.0	2.70	37.80
8	12.6	1.99	25.07
9	17.5	1.58	27.65
10	15.5	1.99	30.84
12	15.5	3.13	48.15
13	13.0	2.50	60.00
16	13.0	2.80	36.40
17	7.7	0.83	6.39
18	12.0	3.13	37.20
27	6.7	6.50	42.90
28	9.5	11.20	616.00
47	12.5	6.00	75.00
49	9.5	5.70	54.15
50	9.3	8.00	74.40
PROTOCOL 1: SWEET BUTTERMILK			
19	10.0	3.30	33.00

A.1: Continued.

Preparation No.	Volume (ml)	Concentration (mM)[a]	Total Gangliosides Present (μ)
20	5.0	2.20	11.00
21	6.3	1.75	11.03
22	6.3	1.53	9.64
25	7.0	0.63	4.41
PROTOCOL 1: YOGURT			
3	20.0	24.30	486.00
8	21.0	9.70	203.70
9	21.0	10.70	224.70
PROTOCOL 2: CULTURED BUTTERMILK			
11	7.1	0.99	6.99
14	16.0	2.00	32.00
15	30.0	1.70	51.00
PROTOCOL 2: SWEET BUTTERMILK			
24	11.0	2.70	29.70
26	7.0	2.90	20.30
PROTOCOL 3: CULTURED BUTTERMILK			
29	44.0	11.20	616.00
37	10.0	1.52	15.20
PROTOCOL 3: SWEET BUTTERMILK			
32	34.0	5.20	176.80
38	22.0	3.88	85.36
PROTOCOL 4: CULTURED BUTTERMILK			
31	6.0	0.40	2.40

A.1: Continued.

Preparation No.	Volume (ml)	Concentration (mM)[a]	Total Gangliosides Present (μ)
35	12.0	1.50	18.00
39	10.0	2.38	23.80
45	5.2	1.50	7.80
46	5.2	0.00	0.00
PROTOCOL 4: SWEET BUTTERMILK			
30	7.0	3.00	21.00
36	9.0	0.90	8.10
40	10.0	2.70	27.00
41	7.3	5.40	39.42
42	8.2	5.70	46.74
43	8.5	5.50	46.75
44	5.7	7.60	43.32
51	5.2	7.85	40.56
52	8.5	5.60	47.60
PROTOCOL 4: YOGURT			
1	6.0	2.70	16.20
2	6.0	3.20	19.20
4	8.5	2.8	23.80
5	19.0	6.25	56.25
6	9.0	1.50	18.75

[a] Micromolar

A.2: Quantitation of crude mixed gangliosides by resorcinol assay.

Protocol	Ganglioside Content (μmol sialic acid/100 6 powder)		
	Cultured Buttermilk	Yogurt	Sweet Buttermilk
1	46.4 ± 25.70	33.3 ± 34.8	304.8 ± 24
2	30.0 ± 22.13	25.0 ± 06.5	–
3	25.0 ± 14.42	71.9 ± 51.1	–
4	16.5 ± 8.13	35.6 ± 13.9	32.0 ± 27

A.3:Qualitative thin-layer chromatography analyses of crude mixed gangliosides from sweet and cultured buttermilk using four different protocols.

Protocol	Mean Relative Prominence of Components			
	NANA[a]	GD_3[b]	Gangliosides	Contaminants
1–Cultured buttermilk	+	–	+	++++++
Sweet buttermilk	+++	+++++	++++	++++++
2–Cultured buttermilk	+	–	–	+++++++
Sweet buttermilk	–	++++	–	++++
3–Cultured buttermilk	++++	++++	++++	++++
Sweet buttermilk	++++	++++	–	++++
4–Cultured buttermilk	+++	+++	+++	++++
Sweet buttermilk	++++	++++	++++	++++

[a] N-acetylneuraminic acid

[b] Disialoganglioside-3

A.4: Summary of the ganglioside content of crude preparations from dairy products.

Dairy Product	Ganglioside Content (nmol/g dry weight)	Product Concentration (μmol/l)
Sweet buttermilk	250-719	21-61
Cultured buttermilk	165-464	21-58
Yogurt	800	114
Whole milk	–	– 5.6

A.5: Summary of the ganglioside content of purified preparations from dairy products and LAB.

Ganglioside Source	Ganglioside Content[a] (nmol/g dry weight)
LAB	
R-39	26.7 ± 8.5
8803	65.0 ± 13.7
Dairy products	
Sweet buttermilk	11.6 ± 8.1
Cultured buttermilk	7.3 ± 5.0
Yogurt	13.2 ± 5.6

[a]All concentrations are based on sialic acid content as determined by resorcinol assay. Ganglioside content (nmol/g dry weight) was calculated using an average ganglioside molecular weight (1,500 g/mol), approximately that of GD_3.

A.6: Inventory of purified ganglioside preparations from dairy products.

Preparation No.	Concentration (mM)	Volume (ml)	Total Present (μmol)
SWEET BUTTERMILK			
1	0.126	34.0	4.300
3	0.126	15.0	1.887
4	0.107	19.0	2.033
5[a]	0.046	19.0	0.874
6	0.580	7.0	4.060
7	0.186	4.9	0.910
8	0.046	6.5	0.299
9[b]	0.080	10.5	0.840
10	0.104	48.4	5.030
11	0.760	10.0	0.760
12	0.315	21.7	6.836
13	0.127	21.1	2.668
14	0.306	11.4	3.488
18	0.334	15.0	5.010
19	0.465	9.0	4.185
24	0.264	5.0	1.320
CULTURED BUTTERMILK			
1	0.023	18.0	0.414
2	0.074	20.0	1.480
3	0.030	7.0	0.212
4	0.154	8.0	1.232
5	0.232	8.0	1.859
6	0.050	3.1	0.155
7	0.034	10.1	0.343
8	0.136	14.8	2.013
9	0.086	6.2	0.533
10	0.136	19.5	2.652
11	0.136	19.7	2.679
12	0.408	8.5	3.468
13	0.082	7.5	0.615
17	0.360	6.2	2.230
18	0.940	5.8	0.545
19	0.316	10.0	3.160

A.6: Continued.

Preparation No.	Concentration (mM)	Volume (ml)	Total Present (μmol)
YOGURT			
1	0.250	24.0	6.000
2	0.180	20.0	3.600
3	0.000	0.0	0.000
4	0.064	16.0	1.024
5	0.015	3.2	0.048
6	0.176	22.3	3.925
7	0.256	8.8	2.258
8	0.406	8.0	3.248
12	0.483	5.4	2.608
18	0.260	4.7	
LACTIC ACID BACTERIA			
B-PP-5 (R-39)	0.032	7.6	0.243
B-PP-6 (R-39)	0.048	6.1	0.293
B-PP-7 (R-39)	0.052	12.0	0.624
B-PP-11 (8803)	0.080	6.8	0.540
EVAPORATED MILK (12 oz/350 ml can)			
EVAP-1	0.372	5.0	1.860
EVAP-2	0.384	6.0	2.304
EVAP-3	0.336	9.0	3.024
EVAP-4	0.440	8.0	3.520
SIMILAC			
SIMI-1	0.056	6.0	0.336
SOY MILK			
SOY-1	0.200[c]	7.8	1.560
SOY-2	0.060[c]	12.0	0.720

[a]Old samples.

[b](.15 M).

[c]TLC plates produced spots that were orange in color, indicating that they probably were not gangliosides.

CHAPTER III

BACTERIOSTATIC EFFECTS OF GANGLIOSIDES AS DETERMINED BY A DOUBLE AGAR OVERLAY ASSAY TECHNIQUE

Abstract

Using a growth-inhibiting assay, the antimicrobial activity of mixed gangliosides extracted from sweet buttermilk and cultured buttermilk, yogurt, and lactic acid bacteria (LAB) against $K88^+$ and $K99^+$ *Escherichia coli (E. coli)* was determined. Screening small concentrations of extracted mixed gangliosides for growth inhibition of enteric pathogens using conventional microbiological methods is tedious and often produces conflicting results (2, 14, 123). A semi-quantitative plate assay was developed to determine the antimicrobial activity of mixed gangliosides. A double agar overlay technique, in which the top layer was a plated lawn of the test pathogen, was the basis for this assay.

Mixed gangliosides were placed into shallow wells that were punched into the pathogen lawn. Zones of clearing were evident around the wells and corresponded to growth inhibition of the pathogenic bacteria. The zones were transitional, but they persisted from 2 to 16 h after incubation. In addition, samples taken from wells and incubated in a minimal medium for up to 16 h produced no bacterial growth.

Mixed gangliosides extracted from sweet buttermilk and cultured buttermilk, yogurt, and LAB were effective in inhibiting the growth of $K99^+$ *E. coli* (strain

431,0101:K30), $K88^+$ *E. coli* (strain 19K974,0141:K85:K88), and *E. coli* 0157:H7 in the range of 0.09 mM to 0.51 mM. Mixed gangliosides extracted from lactic acid bacteria were the most effective in concentrations ranging from .052 mM to .028 mM in producing zones of inhibition. Standard bovine brain gangliosides (BGG) (Sigma Chemical, St. Louis) were the least effective, with a concentration of 8.4 mM being required to produce inhibition zones.

Introduction

Throughout the world, enterotoxigenic *Escherichia coli* (ETEC) is an important cause of neonatal diarrhea in pigs, calves, and lambs, as well as in humans (34, 64, 119, 143). Two virulence determinants are necessary for ETEC to cause diarrhea. The first is enterotoxin production, and the second is pilus production. Pili are hairlike structures present on the outer cell membrane of *E. coli*, which are required for attachment to occur.

Hemagglutination was first recognized as an adhesive property of *E. coli* in the early 1900s. In 1955, it was demonstrated that the presence of pili on the *E. coli* surface was correlated to this adhesive property (5, 33). Antigenically distinct pili present on separate strains of *E. coli* are capable of infecting neonatal calves K99, F41 (19, 92), pigs K99, K88, 987P, F41 (84, 94, 153), lambs K99 (47), and humans (CFA-I and CFA-II) (37, 38, 39, 40). Pili allow *E. coli* to attach to specific receptors on the mucosal surface of the small intestine, which is the initial step in the disease process (56, 64, 119). Present knowledge about the chemical nature of the receptors indicates that they are composed of glycolipids and/or glycoproteins (119). Previous research has indicated that gangliosides, naturally occurring complex sugar-containing glycosphingolipids, which

have sialic acid as an essential component, serve as recognition sites for the attachment of viruses, bacteria, and bacterial toxins (75, 76, 80). This attachment is attributed to the presence of sialic acids and oligosialyl structures in glycolipids that are part of the microbial cell wall (76, 144). There is growing evidence that, because of this binding ability, gangliosides can influence the growth, infectivity, and pathogenicity of microorganisms. The neutralization of *Vibrio cholera* pathogenicity (63, 177) and the inhibition of binding of *Pseudomonas aeruginosa* to mucous (80) are two examples of the antimicrobial activity of gangliosides. Various glycoproteins and glycolipids may be able to competitively inhibit the binding of piliated *E. coli* to brush borders, epithelial cells, or erythrocytes (99, 116, 120, 183). The N-acetylneuramic acid portion appears to play an important role in the inhibition of binding. Previous studies (133) have demonstrated that mixed gangliosides at low levels exhibit bacteriostatic activity against $K88^+$ *E. coli* and $K99^+$ *E. coli, in vitro.* A sensitive plate assay to determine the bacteriostatic activity of mixed gangliosides was developed using a double agar overlay technique.

Materials and Methods

Double Agar Overlay Technique

To test the growth inhibition of enteric pathogens by mixed gangliosides, an assay involving a double agar overlay was developed. MacConkey's minimal medium (Difco Laboratories, Detroit, MI) (1.5%) was used for the base layer, and de Man-Rogosa-Sharpe (MRS) minimal medium (Difco Laboratories, Detroit, MI) (0.9%) , was used for the top layer or lawn (containing the pathogen). Ten to 15 ml of MRS base agar, previously prepared, were heated to boiling, allowed to cool, and poured into a sterile petri dish. Ten

to 15 ml of top agar stored in test tubes were heated to boiling and then cooled to 45^{0}C (in order to avoid the microbial killing reported at higher temperatures of only 50^{0}C [164]). The top agar was inoculated with an enteric pathogen either K88$^+$ *Escherichia coli* (strain 80-022), K99$^+$ *Escherichia coli-45* (strain 455133), or K99$^+$ *Escherichia coli-47* (strain 472304P) provided by the lab of Dr. Robert Holland, Department of Large Animal Clinical Science, Michigan State University or *E. coli* 0157:H7 provided by the lab of Dr. Julius Jackson, Department of Microbiology, Michigan State University. All tests were performed with 24-h cultures of bacteria (10^8/ml), grown in Minca broth with IsoVitaleX (BBL/Becton Dickinson, Cockeysville, MD). The cultures were incubated aerobically at 37°C in a shaking water bath prior to use. The pathogens and top agar were then gently titrated to encourage even distribution, and poured over the base agar. After the agar congealed, wells (8 mm in diameter and approximately 4mm in depth) were punched (using a brass cork corer) into only the top agar. Seventy-five mM of various mixed gangliosides were added to wells. The plates with innoculated mixed gangliosides were incubated at 34^{0}C with growth inhibition zones measured at designated intervals.

Zones of clearing, or growth-inhibition zones, were measured and designated qualitatively as follows: clear–no apparent growth, cloudy–a small amount of growth, opaque–some growth but much less than outside of the zone, and growth–no apparent inhibition. designations of clear to opaque. Zones of clearing were measured from the edge of the well to the outer edge of the zone, in millimeters (mm). Growth within the zone was also designated as clear (no apparent growth), cloudy (a little growth, but printed text could be read when placed under the petri dish), opaque (some growth, but printed text could not be read when placed under the petri dish), and growth (no apparent

growth inhibition). The zone sizes and designations of clarity were averages (a minimum of three replications at each concentration), with the clarity being reproducible (no averaging of clear and opaque to produce cloudy was done). Attempts were made to reduce the zones of clearing by1/2 and 1/4, using1/2 and 1/4 the amount of samples that produced the original inhibition zones. Negative controls used were autoclaved gangliosides, autoclaved distilled water, and empty wells.

Gangliosides used for inhibition were bovine brain gangliosides (Sigma Chemical); two starter cultures one designated 8803 from a cultured buttermilk source (Darigold, Seattle, WA) and the second designated R-39 (Chris Hansen's laboratory, Milwaukee, WI), which is used in the production of yogurt supplied to the Michigan State University Dairy Plant; and lactic acid bacteria gangliosides obtained from a buttermilk starter culture (Darigold, Seattle, WA). Sweet and cultured buttermilk, yogurt and lactic acid bacteria gangliosides were extracted in the lab of Dr. Kathleen Moore, Department of Chemistry at Oakland University, Michigan. The ganglioside extraction protocol used was based on a Folch-like extraction as modified by Svennerholm and Friedman (168). Modifications to this procedure included: an acetone pre-extraction step to remove natural lipids; dialysis and lyophilization; the resuspension of powdered milk or yogurt in water which was then extracted with a series of chloroform/methanol solutions and subsequently partitioned (75, 168). Ultimately, an extraction and diethylaminoethyl (DEAE)-Sephadex A-25 (Sigma Chemical, St. Louis, MO) chromatography procedure as described by Bonafede et al. (14) yielded what were termed purified preparations. Thin Layer Chromatography (TLC) was performed using silica gel 60 TLC precoated plates (glass plates 10 x 10cm, Merck) for ganglioide quantitation. The plates were activated at

100° C and ganglioside preparations and ganglioside and N-acetylneuraminic acid (NANA) standards were spotted on silica gel G plates and placed in a chamber containing chloroform: methanol:0.25% $CaCL_2$ solution (30:18:4). Plates were developed in the chambers for twice the time it took the solvent to reach the top of the plate. Plates were then removed, dried, sprayed with resorcinol-HCL for visualization, and heated at 110°C for 15 min. Sialic-acid-containing material gave blue spots; organic material lacking sialic acid charred brown. Densitometry of TLC plates was performed with a Shimadzu Dual-Wavelength TLC-Scanner CS930. The chromatograms were obtained by single wavelength zig-zag scanning of each lane at 620 nm (beam dimension: 0.4 X 0.4mm) in the reflection mode (88, 168). The purified preparations yielded blue bands of varying patterns. These purified mixed ganglioside preparations, were used for the growth inhibition studies.

Bacterial-Growth Studies

The concentration of viable cells is sometimes roughly estimated by a method designated as the Most Probable Numbers (MPN) (30). This procedure is based on a mathematical calculation of multiple cultures that fail to show growth in a series of dilution tubes containing an appropriate medium. As a modification of the MPN method, ten µl of mixed gangliosides were recovered from sample wells where zones of clearing were evident. Three replicates are the minimum performed for bacterial-growth studies. Ten-microliter aliquots were recovered at 2, 4, 6, and 8 hour intervals. Wells from which samples were obtained were not used for zones-of-clearing measurements because the reduced amounts in the wells may have decreased the inhibition zones. The 10 µl

aliquots were then inoculated into 5-ml tubes of 1/2 de Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories, Detroit MI) with 1% lactose (Sigma Chemical, St. Louis MO) and incubated at 37^0C for the intervals indicated. Using a spectrophotometer, optical density readings were taken at 2-, 4-, 8-, 12-, and 16-h intervals to test for any growth inhibition.

Results

A sensitive plate assay to determine the bacteriostatic activity of mixed gangliosides was developed using a double agar overlay technique. A photograph of this assay is depicted in Figure III.1. The results of the double agar overlay technique with enteric pathogens are presented in Figures III.2 through III.13. The ability of mixed gangliosides to inhibit growth varied with the ganglioside concentration, the ganglioside source, and the specific pathogen being tested. Purified mixed gangliosides were the only samples tested experimentally. Early crude ganglioside extractions were used with this technique, but the purified gangliosides produced more consistent reproducible results due to the purification process. Purified mixed gangliosides from sweet buttermilk, cultured buttermilk, yogurt, and lactic acid bacteria with ganglioside concentrations in the range of 0.018mM to 19.4mM were effective in inhibiting the growth of $K99^+$ *E. coli,* $K88^+$ *E. coli,* and *E. coli* 0157:H7.

Zones of clearing were transitory, with zones fading from clear to cloudy, turning opaque, and gradually disappearing as bacterial growth covered the area. Depending on the growth inhibition, some zones were still apparent after 14 to 18 h of incubation. Zones appeared most evident after 4 to 6 h incubation, with the photograph in Figure III.1 being taken at 5 h of incubation. As ganglioside concentration increased, the zone's

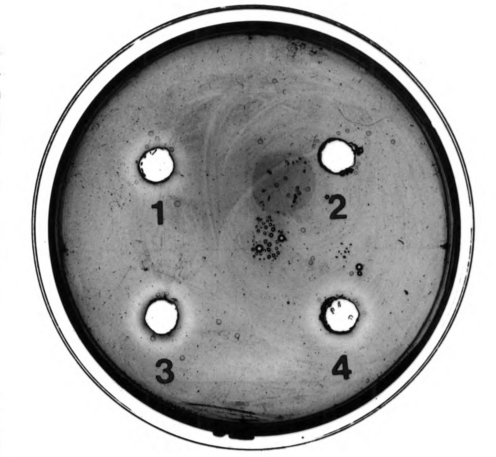


Figure III.1: Bacteriostatic effects of mixed gangliosides from sweet buttermilk, lactic acid bacteria, and bovine brain gangliosides on K88$^+$ *E. coli* using a double agar overlay technique.[a]

[a]Well 1 contains bovine brain gangliosides (19 mM), Well 2 contains sterile water, Well 3 contains lactic acid bacteria mixed gangliosides(0.32 mM), and Well 4 contains sweet buttermilkmixed gangliosides (0.58 mM).

[b]Five-hour-growth K88$^+$ *E. coli* bacteria was used.

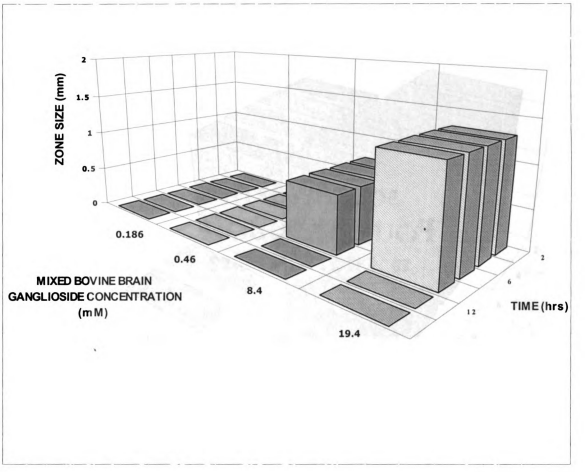


Figure III.2: Formation of inhibition zones around wells containing varying concentrations of mixed bovine brain gangliosides. Each well was produced in a lawn of K88$^+$ *Escherichia coli* and contained 1 ml of ganglioside extract per well. Ganglioside quantitation required that preparations and standards be spotted on silica gel G plates (Merck), placed in a chamber containing chloroform: methanol:0.25% $CaCL_2$ solution (30:18:4); developed; removed, dried, sprayed with resorcinol-HCL for visualization, and heated at 110°C for 15 min. Zones of inhibition were measured at 2, 4, 6, 8, 12 and 14 hour intervals and values are means with a minimum of three replications at each concentration.

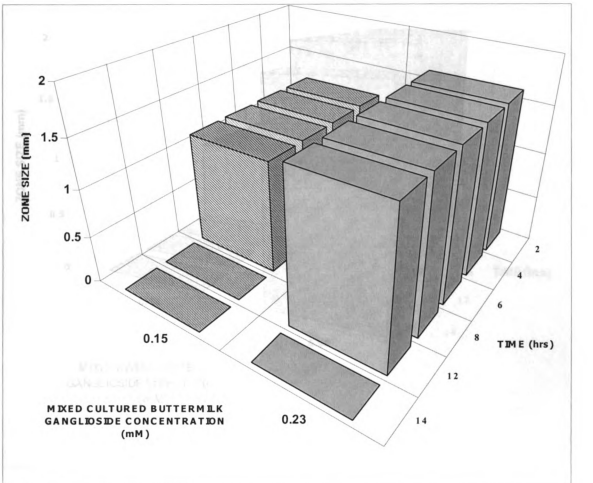


Figure III.3: Formation of inhibition zones around wells containing varying concentrations of mixed cultured butter milk gangliosides. Ganglioside quantitation required that preparations and standards be spotted on silica gel G plates (Merck); placed in a chamber containing chloroform: methanol:0.25% $CaCL_2$ solution (30:18:4); developed; removed; dried; sprayed with resorcinol-HCL for visualization; and heated at 110°C for 15 min. Each well was produced in a lawn of $K88^+$ *Escherichia coli* and contained 1 ml of ganglioside extract per well. Zones of inhibition were measured at 2, 4, 6, 8, 12 and 14 hour intervals and values are means with a minimum of three replications at each concentration.

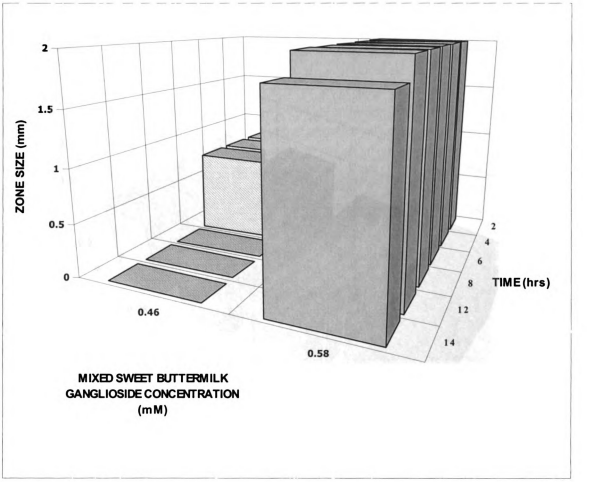


Figure III.4: Formation of inhibition zones around wells containing varying concentrations of mixed sweet buttermilk gangliosides. Ganglioside quantitation required that preparations and standards be spotted on silica gel G plates (Merck); placed in a chamber containing chloroform: methanol:0.25% $CaCL_2$ solution (30:18:4); developed; removed; dried; sprayed with resorcinol-HCL for visualization; and heated at 110°C for 15 min. Each well was produced in a lawn of $K88^+$ *Escherichia coli* and contained 1 ml of ganglioside extract per well. Zones of inhibition were measured at 2, 4, 6, 8, 12 and 14 hour intervals and values are means with a minimum of three replications at each concentration.

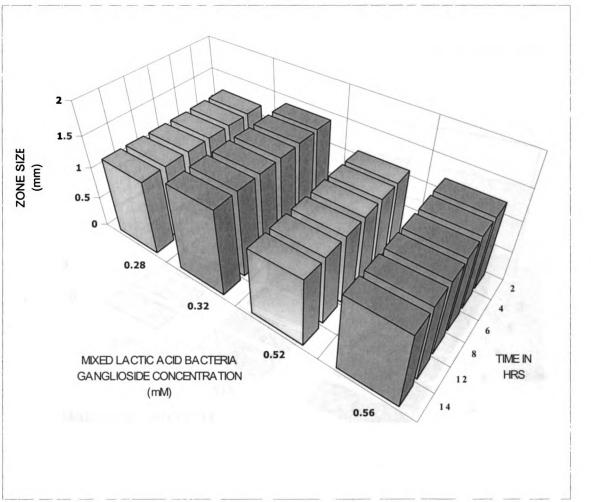


Figure III.5: Formation of inhibition zones around wells containing varying concentrations of mixed lactic acid bacterial gangliosides. Ganglioside quantitation required that preparations and standards be spotted on silica gel G plates (Merck); placed in a chamber containing chloroform: methanol:0.25% $CaCL_2$ solution (30:18:4); developed; removed; dried; sprayed with resorcinol-HCL for visualization; and heated at 110°C for 15 min. Each well was produced in a lawn of $K88^+$ *Escherichia coli* and contained 1 ml of ganglioside extract per well. Zones of inhibition were measured at 2, 4, 6, 8, 12 and 14 hour intervals and values are means with a minimum of three replications at each concentration.

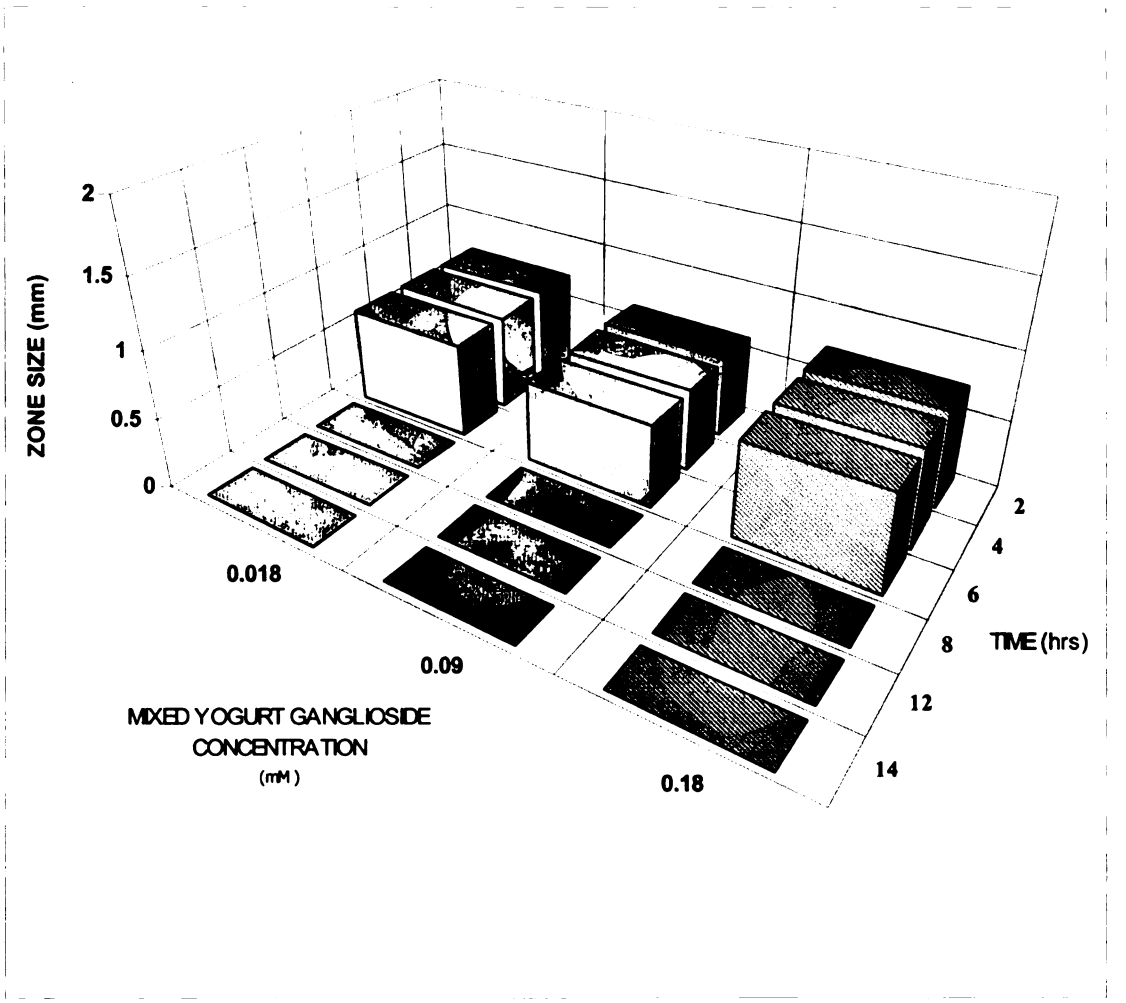


Figure III.6: Formation of inhibition zones around wells containing varying concentrations of mixed yogurt gangliosides. Ganglioside quantitation required that preparations and standards be spotted on silica gel G plates (Merck); placed in a chamber containing chloroform: methanol:0.25% $CaCL_2$ solution (30:18:4); developed; removed; dried; sprayed with resorcinol-HCL for visualization; and heated at 110°C for 15 min. Each well was produced in a lawn of $K88^+$ *Escherichia coli* and contained 1 ml of ganglioside extract per well. Zones of inhibition were measured at 2, 4, 6, 8, 12 and 14 hour intervals and values are means with a minimum of three replications at each concentration.

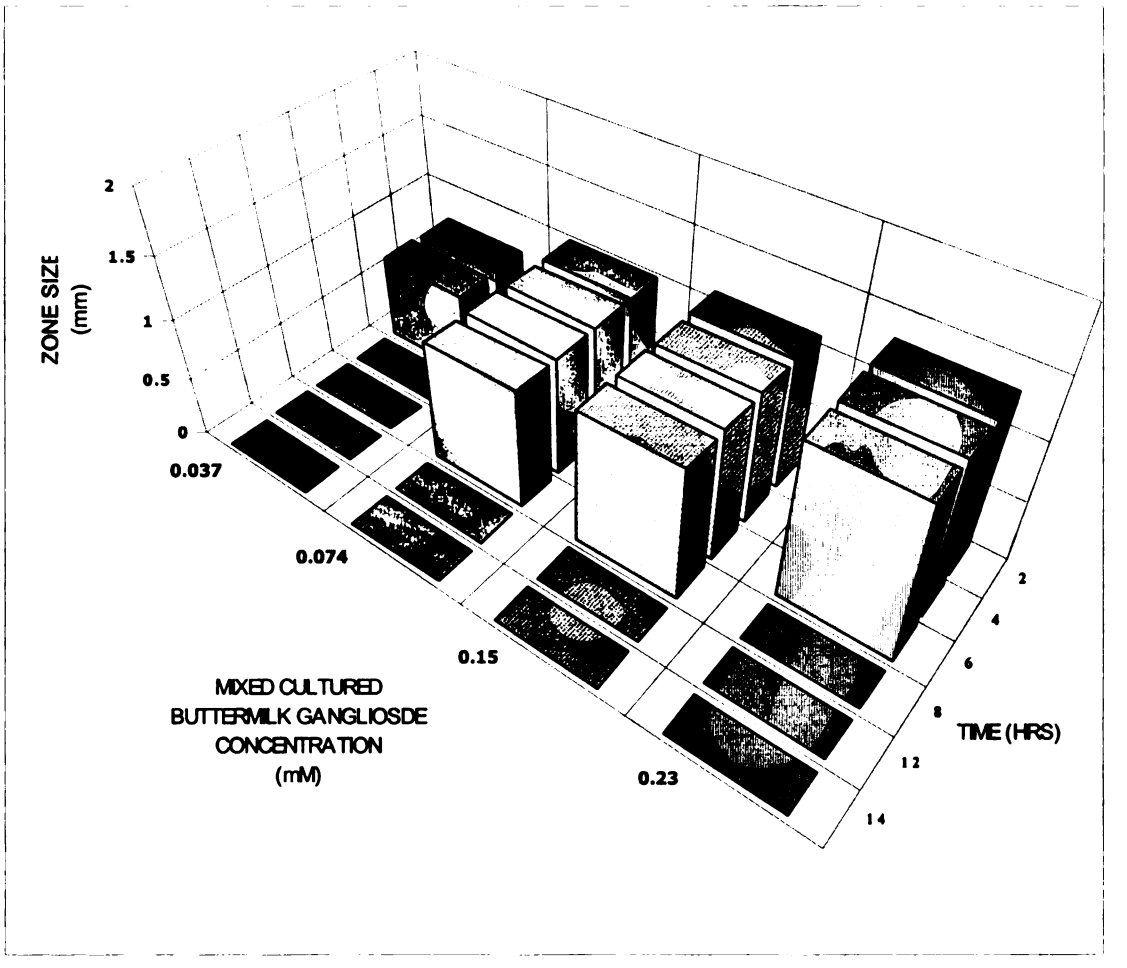


Figure III.7: Formation of inhibition zones around wells containing varying concentrations of mixed cultured buttermilk gangliosides. Ganglioside quantitation required that preparations and standards be spotted on silica gel G plates (Merck); placed in a chamber containing chloroform: methanol:0.25% $CaCL_2$ solution (30:18:4); developed; removed; dried; sprayed with resorcinol-HCL for visualization; and heated at 110°C for 15 min. Each well was produced in a lawn of $K99^+$ *Escherichia coli* and contained 1 ml of ganglioside extract per well. Zones of inhibition were measured at 2, 4, 6, 8, 12 and 14 hour intervals and values are means with a minimum of three replications at each concentration.

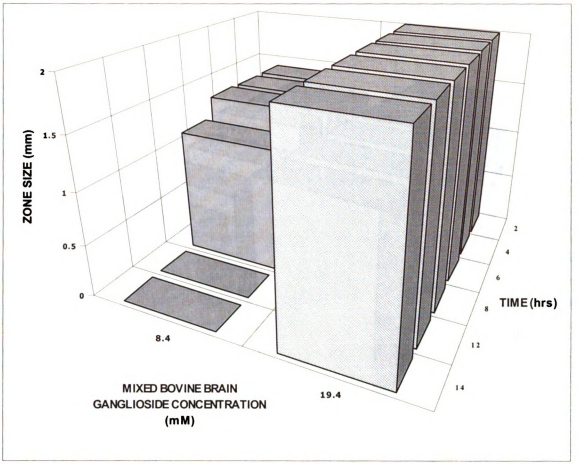


Figure III.8: Formation of inhibition zones around wells containing varying concentrations of mixed bovine brain gangliosides. Ganglioside quantitation required that preparations and standards be spotted on silica gel G plates (Merck); placed in a chamber containing chloroform: methanol:0.25% $CaCL_2$ solution (30:18:4); developed; removed; dried; sprayed with resorcinol-HCL for visualization; and heated at 110°C for 15 min. Each well was produced in a lawn of $K99^+$ *Escherichia coli* and contained 1 ml of ganglioside extract per well. Zones of inhibition were measured at 2, 4, 6, 8, 12 and 14 hour intervals and values are means with a minimum of three replications at each concentration.

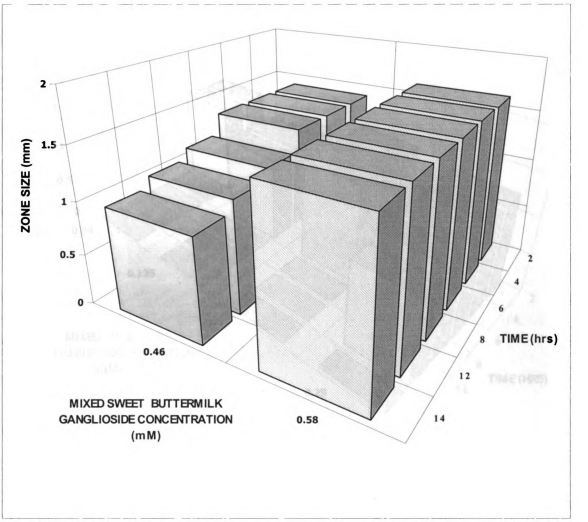


Figure III.9: Formation of inhibition zones around wells containing varying concentrations of mixed sweet buttermilk gangliosides. Ganglioside quantitation required that preparations and standards be spotted on silica gel G plates (Merck); placed in a chamber containing chloroform: methanol:0.25% $CaCL_2$ solution (30:18:4); developed; removed; dried; sprayed with resorcinol-HCL for visualization; and heated at 110°C for 15 min. Each well was produced in a lawn of $K99^+$ *Escherichia coli* and contained 1 ml of ganglioside extract per well. Zones of inhibition were measured at 2, 4, 6, 8, 12 and 14 hour intervals and values are means with a minimum of three replications at each concentration.

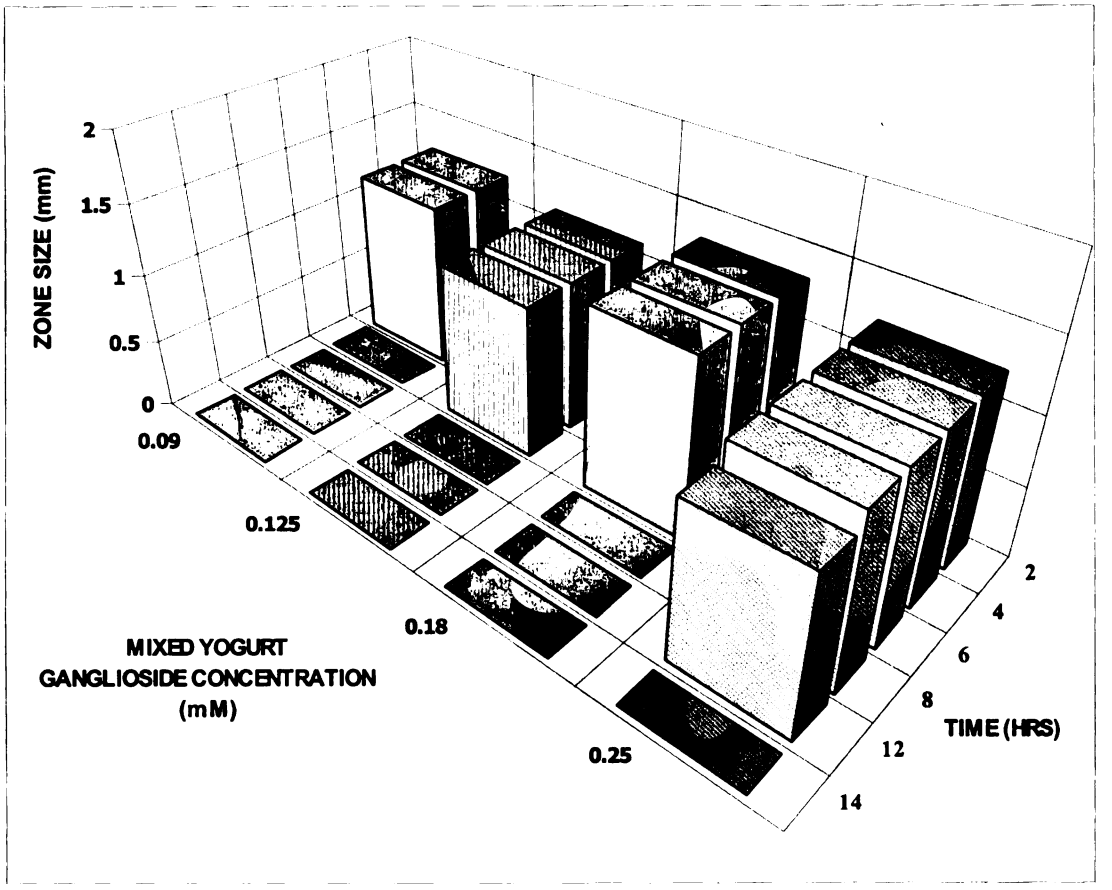


Figure III.10: Formation of inhibition zones around wells containing varying concentrations of mixed yogurt gangliosides. Ganglioside quantitation required that preparations and standards be spotted on silica gel G plates (Merck); placed in a chamber containing chloroform: methanol:0.25% $CaCL_2$ solution (30:18:4); developed; removed; dried; sprayed with resorcinol-HCL for visualization; and heated at 110°C for 15 min. Each well was produced in a lawn of $K99^+$ *Escherichia coli* and contained 1 ml of ganglioside extract per well. Zones of inhibition were measured at 2, 4, 6, 8, 12 and 14 hour intervals and values are means with a minimum of three replications at each concentration.

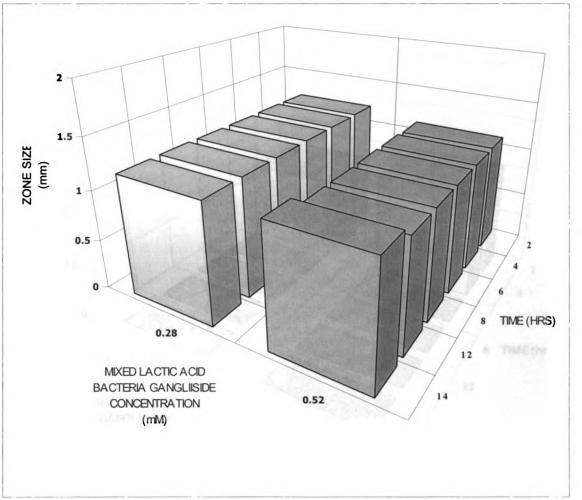


Figure III.11: Formation of inhibition zones around wells containing varying concentrations of lactic acid bacterial gangliosides. Ganglioside quantitation required that preparations and standards be spotted on silica gel G plates (Merck); placed in a chamber containing chloroform: methanol:0.25% $CaCL_2$ solution (30:18:4); developed; removed; dried; sprayed with resorcinol-HCL for visualization; and heated at 110°C for 15 min. Each well was produced in a lawn of $K99^+$ *Escherichia coli* and contained 1 ml of ganglioside extract per well. Zones of inhibition were measured at 2, 4, 6, 8, 12 and 14 hour intervals and values are means with a minimum of three replications at each concentration.

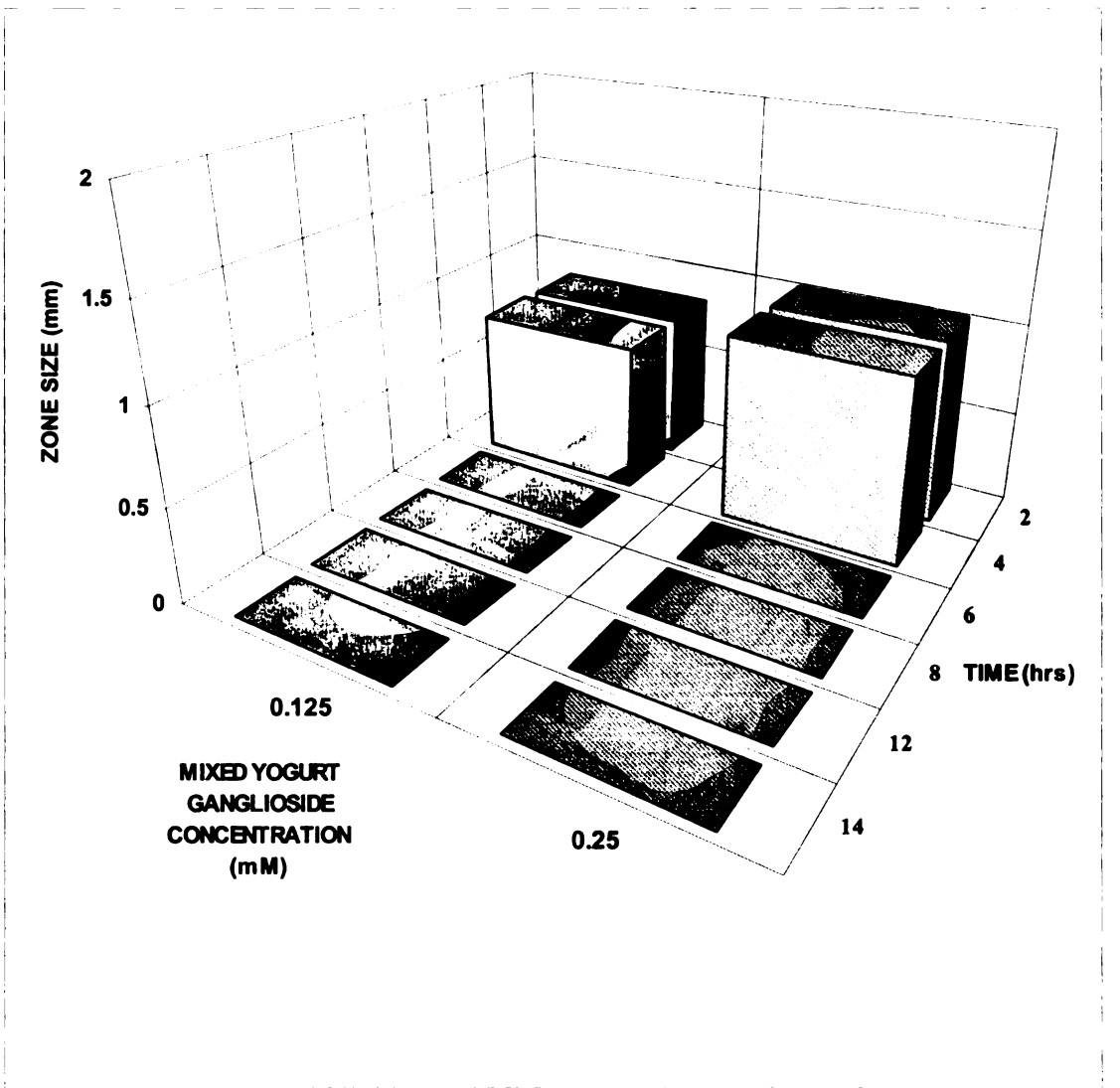


Figure III.12: Formation of inhibition zones around wells containing varying concentrations of mixed yogurt gangliosides. Ganglioside quantitation required that preparations and standards be spotted on silica gel G plates (Merck); placed in a chamber containing chloroform: methanol:0.25% $CaCL_2$ solution (30:18:4); developed; removed; dried; sprayed with resorcinol-HCL for visualization; and heated at 110°C for 15 min. Each well was produced in a lawn of *Escherichia coli* 0157:H7 and contained 1 ml of ganglioside extract per well. Zones of inhibition were measured at 2, 4, 6, 8, 12 and 14 hour intervals and values are means with a minimum of three replications at each concentration.

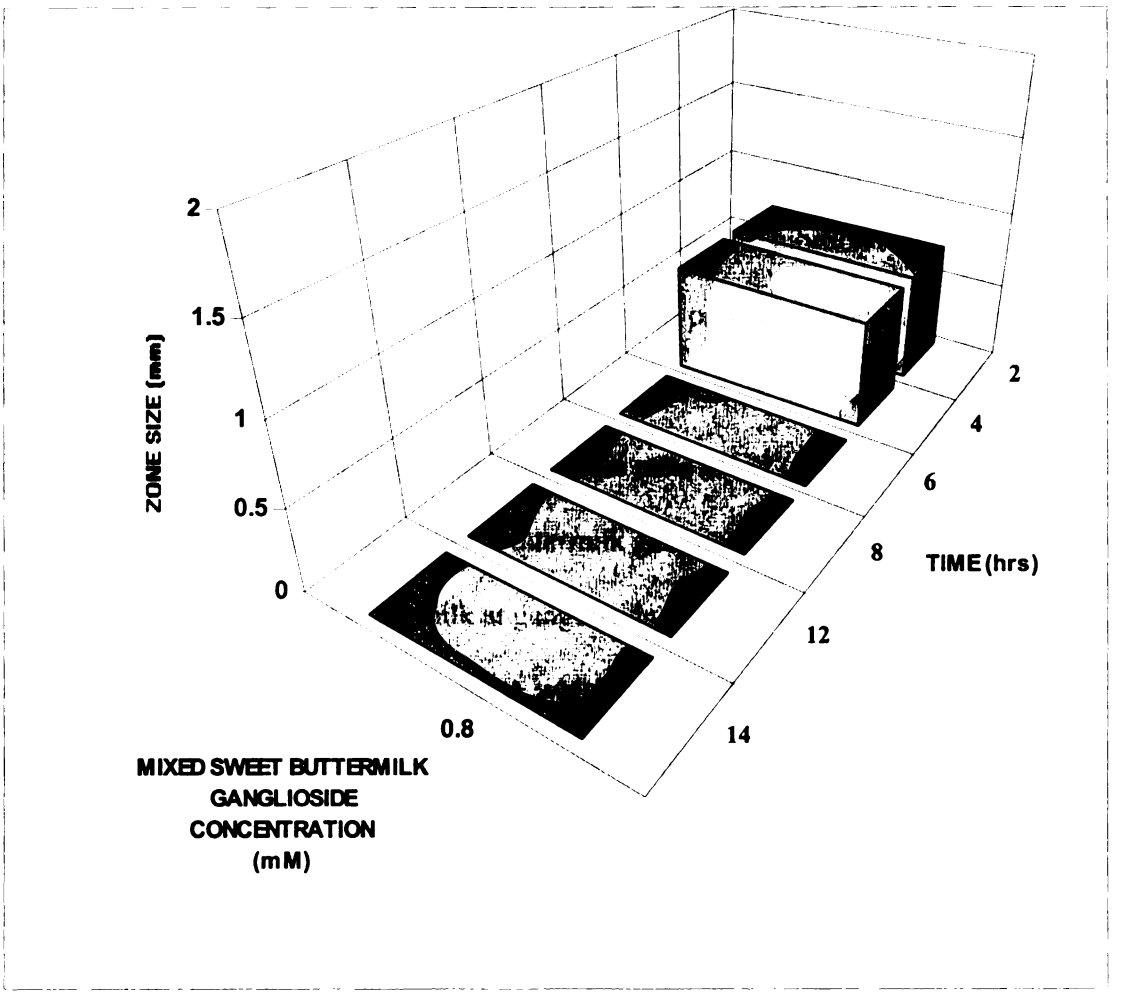


Figure III.13: Formation of inhibition zones around wells containing varying concentrations of mixed sweet buttermilk gangliosides. Ganglioside quantitation required that preparations and standards be spotted on silica gel G plates (Merck); placed in a chamber containing chloroform: methanol:0.25% $CaCL_2$ solution (30:18:4); developed; removed; dried; sprayed with resorcinol-HCL for visualization; and heated at 110°C for 15 min. Each well was produced in a lawn of *Escherichia coli* 0157:H7 and contained 1 ml of ganglioside extract per well. Zones of inhibition were measured at 2, 4, 6, 8, 12 and 14 hour intervals and values are means with a minimum of three replications at each concentration.

appearance remained clearer for a longer time. Bovine brain gangliosides at a concentration of 19.4 mM produced a clear zone, which remained clear for 6 h, gradually progressing to growth by 12 h (Figure III.2). However, bovine brain gangliosides at .46 mM ganglioside concentration produced no apparent zone of inhibition (Figure III.2). Concentrations of LAB gangliosides as low as 0.28 mM produced zones that remained clear for up to 14 h (Figures III.5 and III.11). Sweet buttermilk ganglioside concentrations as minute as 0.8 mM produced some zone effect (cloudy) (Figure III.13), with clear zones being achieved at sweet buttermilk ganglioside concentrations of .58 mM (Figure III.4). Cultured buttermilk at ganglioside concentrations of .037 mM also produced observable results (cloudy zones), which remained cloudy for up to 4 h (Figures III.6 and III.10) persisted for 6 h in some cases (Figure III.7). Yogurt samples with ganglioside concentrations as low as .09 mM produced zones that were cloudy in appearance and The clear zones appeared more frequently and persisted longer on lawns of $K99^+$ *E. coli* (Figures III.7 through III.11) as compared to $K88^+$ *E. coli* (Figures III.2 through III.6). This was true for all ganglioside sources. When testing similar ganglioside concentrations, there did not appear to be any discernible difference between lawns of *E. coli* 0157:H7 (Figure III.12 and III.13) and those of either $K99^+$ *E. coli* (Figures III.7 through III.11) or $K88^+$ *E. coli* (Figures III.2 through III.6). If plates were removed from the incubator and placed at 5^0C, zones remained for all samples indefinitely, (plates were refrigerated for one week) because the bacterial growth was apparently retarded by the low temperature.

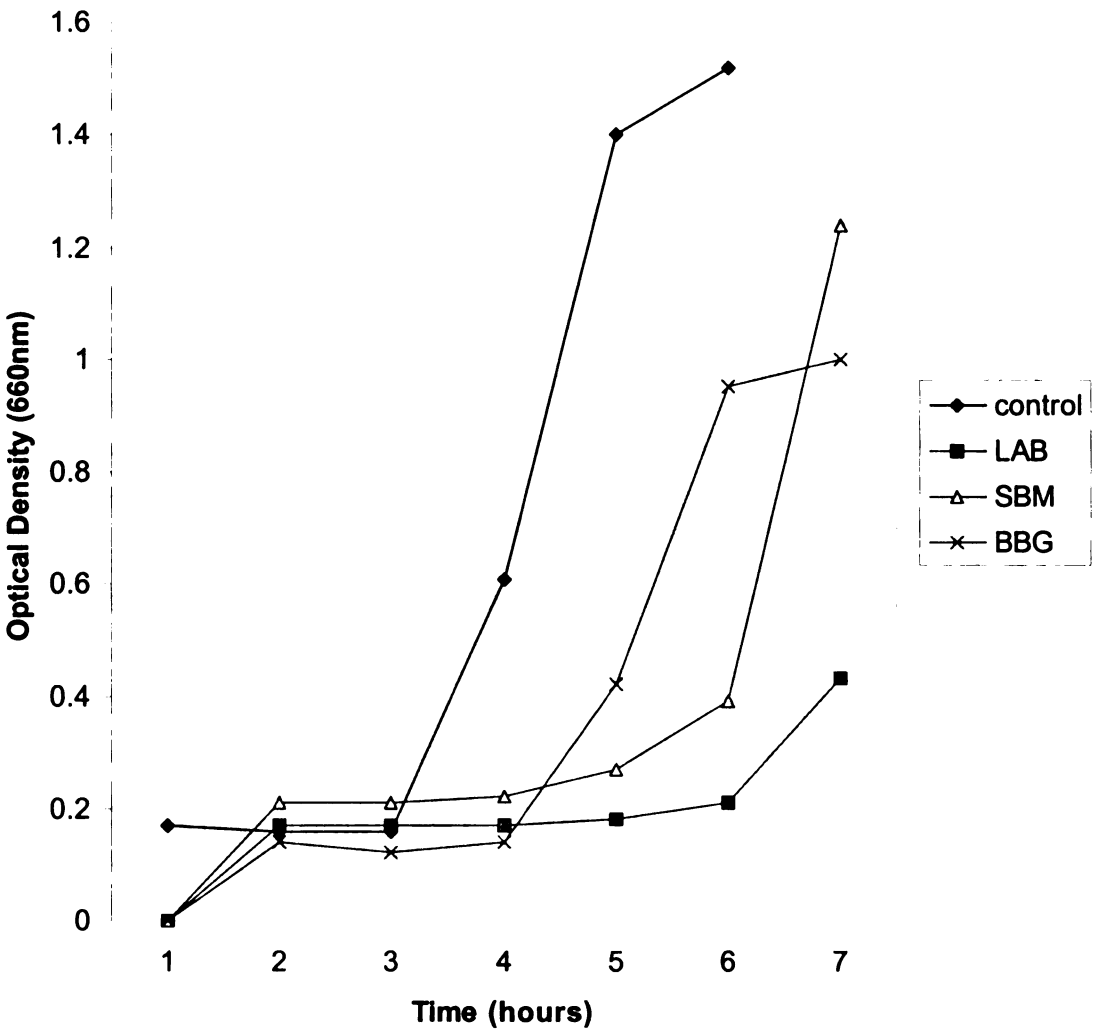


Fig. III.14: Growth inhibition of K99$^+$ *Escherichia coli* (*E. Coli*). Ten microliter samples of mixed bovine brain gangliosides (BBG), mixed lactic acid bacterial gangliosides (LAB), mixed sweet buttermilk gangliosides (SWB) and sterile water (control) were extracted from double agar overlay wells which exhibited zones of inhibition, and placed in 5ml of K99$^+$ *E. coli.* K99$^+$ *Escherichia coli* growth occurred for two hours (h) prior to ganglioside addition. Growth inhibition was measured by optical density readings conducted at 2h intervals for 16 h at performed at 660 nanometers.

In order to determine if a bacteriostatic effect could be detected through growth turbidity, gangliosides which produced zones of inhibition were extracted and inoculated into samples of $K99^+$ *E. coli*. Ten-microliter samples of gangliosides were extracted from specific wells that produced zones of clearing at 2-, 4-, and 6-h intervals. The results of ganglioside incubation are illustrated in Figure III.14. Each value represents an average of three replications. Bacterial growth was determined by turbidity readings using a spectrophotometer at 660 nm. Comparing readings at initial incubation to 16 h after incubation, all samples extracted from the wells after 8h produced bacterial growth, consistent with 24-h growth samples (Figure III.14). Mixed gangliosides, from lactic acid bacteria, sweet buttermilk and bovine brain gangliosides retarded growth for 5h with slow growth apparent for 6h with lactic acid bacteria and sweet buttermilk (Figure III.14). Growth retardation was still evident for LAB at 7h (Figure III.14). Negative controls produced bacterial growth when sampled at 2-, 4-, and 6-h intervals (Figure III.14).

Discussion

The ability of lactobacillus strains to interfere with the growth of intestinal pathogens has been previously investigated (23, 27). The mode of this inhibition has been suggested to be attributed to lactic acid production (90), and more recently an effect termed bacterial coaggregation (22). The research which produced the previously mentioned results, utilized procedures which were labor intensive and often cost prohibitive. The ability to screen low concentrations of mixed gangliosides for growth inhibition of enteric pathogens using non-conventional microbial methods could prove to

be a valuable research tool. Developing a reliable procedure for screening low concentrations of mixed gangliosides for growth inhibition did prove to be a challenge. Approaches that proved to be unsuccessful included streaking ganglioside solutions over inoculated plates, soaking filter-paper disks with gangliosides and placing them on pathogen-inoculated plates, inoculating actively growing cultures with gangliosides, and incubating pathogens with ganglioside preparations before plating. The double agar overlay technique produced results that confirmed the applicability of the method for rapidly screening small quantities of gangliosides and determining their growth-inhibiting capabilities. Mixed bovine brain gangliosides required higher concentrations to produce similar zones of clearing (Figure III.2 and III.8) when compared to gangliosides from lactic acid bacteria (Figure III.5 and III.11), sweet buttermilk (Figure III.4 and III.9), yogurt (Figure III.6 and III.10) and cultured buttermilk (Figure III.3 and III.7). The lowest concentration of bovine brain gangliosides to produce a detectable zone of inhibition was 8.4 mM (Figure III.2). In contrast, a concentration of only 0.46 mM sweet buttermilk was effective in inhibiting growth. Sweet buttermilk was more effective than cultured buttermilk at lower concentrations (Figures III.3 and III.4). Gangliosides extracted from lactic acid bacteria were the most effective, with concentrations ranging from 0.52 to 0.28 mM producing clear zones that persisted for up to 8 h (Figure III.5). The negative controls used were autoclaved samples of gangliosides, sterile water, and empty wells. No zones of inhibition were observed surrounding any of the negative control wells, indicating that the gangliosides were solely responsible for the effect. Recovering sufficient quantities of gangliosides to accurately measure their effects was a continuing challenge during this research project. In the initial studies,

ganglioside source and concentration were compared for inhibition ability and for quantification with regard to ganglioside concentrations. Dose-responsive studies were later performed that included dilution factors of 1/2 and 1/4 in an attempt to determine whether growth inhibitions could be reduced by these amounts. The ability to curtail bovine brain ganglioside (BBG) inhibition by approximately 1/2 was demonstrated by reducing BBG content from 19.4 mM to 8.4 mM. Other ganglioside sources, however, did not demonstrate this effect. In addition, BBG required higher concentrations to produce similar zones of clearing when compared to gangliosides from lactic acid bacteria and sweet and cultured buttermilks. The lowest concentration of BBG to produce a zone of inhibition was 8.4 mM. A concentration of 0.46 mM sweet buttermilk was effective in producing inhibition. Sweet buttermilk was more effective than cultured buttermilk at lower concentrations. This effect could be attributed to ganglioside composition, which has been demonstrated to play an important role with respect to growth inhibition. Sweet buttermilk may contain specific cell membrane receptors for *K99*$^{+}$ *E. coli*. Gangliosides extracted from lactic acid bacteria were the most effective when taken from wells at 2- and 4-h intervals. Sweet buttermilk, cultured buttermilk, and yogurt mixed gangliosides produced varying results. In general, as ganglioside concentration was increased by 1/2 or 1/4, the zones of clearing were increased by these same increments. It was demonstrated, however, that there was a concentration effect for some gangliosides. In general, the higher the ganglioside concentration of the BBG, sweet buttermilk, cultured buttermilk, and yogurt sample, the larger, more persistent, and clearer the zones appeared. BBG, however, was the least effective inhibitor. Since BBG was likely the purest of the gangliosides because it was commercially prepared by Sigma

Chemical; the possibility did exist that other factors were present in the ganglioside extractions and contributed to the bacteriostatic effects noted.

It is important to reemphasize that ganglioside composition pays an important role with respect to growth inhibition and that certain gangliosides have been identified as the specific cell membrane receptors, (14, 85, 87). The differences in inhibition rates can possibly be attributed to ganglioside sample composition. Additionally, not all gangliosides have demonstrated growth inhibition capabilities. Sample viscosity could be an extra factor in determining the size and clarity of zones produced. Some samples, especially yogurt, were more viscous than others due to their initial density prior to extraction. The more vicious samples were apparently unable to diffuse into the pathogen-containing lawn at a rate that could significantly retard bacterial growth. This may explain why, even at high concentrations, yogurt gangliosides produced only cloudy zones. Although zones of clearing are transitory, the fact that bacterial growth was not observed at intervals as great as 24 h for some mixed gangliosides does suggest the possibility that the effects may have been bacteriostatic in nature. Although the determination of zones as clear, cloudy, or opaque did not always allow for the most accurate description, it did make comparisons between zones more quantifiable.

In general, the growth of bacterial samples in the presence of gangliosides taken from wells mimicked the ability of these mixed gangliosides to form zones. The larger and clearer the zones, the more reduced was the growth of bacteria taken from the wells, which seemed to indicate a bacteriostatic effect. The gangliosides from LAB were effective in eliciting no bacterial growth in minimal medium at 2- and 4-h intervals. Whereas growth ultimately did occur in all other samples, it was greatly retarded in many

of them. The negative controls produced bacterial growth similar to the normal 24-h growth of bacteria.

Although the specific mode of action of growth inhibition by gangliosides is unknown, trisialoganglioside has been shown to inhibit the tyrosine kinase of epidermal growth factor receptor, inhibiting cell growth and proliferation (171) . *In vivo* the pathogenicity of entertoxigenic *E. coli* in infectious diarrhea is due in large part to the action of enterotoxins (28, 111). It is also known that adhesion of bacteria to the host plays an important role in bacterial virulence (39). Adhesion of bacteria is an early process in the development of an infection which involves binding of bacterial pili to intestinal glycolipid receptors (111). Bacteria bind to the intestinal mucosa to begin the colonization process and elicit disease. Any substances that affect the adhesion of entertoxigenic *E. coli* to the intestinal mucosa cells will reduce the pathogenicity of microorganisms(39, 111). The opportunity to use this newly developed technique in determining the growth inhibition of small quantities of mixed gangliosides and other materials is a realistic and achievable goal.

CHAPTER IV

INHIBITION OF ADHESION OF K99$^+$ *Escherichia coli (E. coli)* AND COLONIZATION FACTOR ANTIGENS-I$^+$ (CFA-I$^+$) BY PURIFIED MIXED GANGLIOSIDES EXTRACTED FROM LACTIC ACID BACTERIA, BOVINE MILK FAT, AND CULTURED DAIRY FOODS

Abstract

A lipid component of milk fat, referred to as gangliosides, which is also present in cultured dairy food products and lactic acid bacteria (LAB) used to produce these dairy food products, possesses antimicrobial activity. Mixed gangliosides recovered and purified from LAB and cultured dairy products were effective in inhibiting the pilus-mediated adhesion of K99$^+$ *Escherichia coli (E. coli)* to sheep and horse erythrocytes, and colonization factor antigen-I (CFA-I$^+$) *E. coli* to chicken and cow erythrocytes. Ganglioside inhibition of K99$^+$ *E. coli* and CFA-I$^+$-mediated adhesion was assessed using a mannose-resistant hemagglutination assay. Bacteria were preincubated with mixed gangliosides before erythrocyte addition, and nonagglutinated erythrocytes were counted electronically with an electron particle counter (Coulter Counter). Using horse and sheep erythrocytes with K99$^+$ *E. coli,* hemagglutination was inhibited by 19% to 100% in ganglioside-treated samples (concentrations of 0.018 mM to 0.074 mM). Incubating CFA-I$^+$ with cow and chicken erythrocytes and mixed gangliosides ranging from 0.026 mM to 0.176 mM produced hemagglutination inhibition ranging from 41% to 81%. These hemagglutination-inhibition results suggest that mixed gangliosides extracted from

cultured dairy foods and LAB could play an important role in limiting the adhesion of enterotoxigenic *E. coli* ($K99^+$ and $CFA\text{-}I^+$) to the intestinal mucosa, thus preventing or lessening the effects of diarrhea.

Introduction

Throughout the world, enterotoxigenic *Escherichia coli* (ETEC) is an important cause of neonatal diarrhea in pigs, calves, and lambs, as well as in humans (34, 64, 119, 143). Two virulence determinants are necessary for ETEC to cause diarrhea. The first is enterotoxin production, and the second is pilus production. Pili are hairlike structures present on the outer cell membrane of *E. coli.*

Hemagglutination was first recognized as an adhesive property of *E. coli* in the early 1900s. In 1955, it was demonstrated that the presence of pili on the *E. coli* surface was correlated to this adhesive property (33). Antigenically distinct pili present on separate strains of *E. coli* are capable of infecting neonatal calves K99, F41 (19, 92), pigs K99, K88, 987P, F41 (84, 94, 153), lambs K99 (47, 92), and humans (CFA-I and CFA-II) (37, 38, 39, 40). Pili allow *E. coli* to attach to specific receptors on the mucosal surface of the small intestine, which is the initial step in the disease process (56, 64, 119). Present knowledge about the chemical nature of the receptors indicates that they are composed of glycolipids and/or glycoproteins. Various glycoproteins and glycolipids may be able to competitively inhibit the binding of piliated *E. coli* to brush borders, epithelial cells, or erythrocytes (99, 116, 120, 183). The N-acetylneuraminic acid portion appears to play an important role in the inhibiting bacterial adhesion to epithelia thereby preventing bacterial infection at the stage of bacterial colonization (116, 120, 183).

Hemagglutination inhibition of $K99^+$ ETEC has been demonstrated with a glycoconjugate resembling the monosialoganglioside-2 (GM_2) (93). The carbohydrate moiety of the sheep erythrocyte receptor also appears to resemble ganglioside GM_2. The hemagglutination-inhibition assay mimics ETEC pili binding to the intestinal mucosa and therefore, could serve as a good test system for evaluating gangliosides.

Materials and Methods

Source of Gangliosides

Mixed ganglioside extractions and purifications were performed in the laboratory of Dr. Kathleen Moore, Department of Chemistry, Oakland University. The purifications were conducted on lactic acid bacteria (LAB) obtained from a buttermilk starter culture (Darigold, Seattle, WA), sweet and cultured powdered buttermilk (Darigold, Seattle, WA), and plain cultured yogurt (MSU Dairy Plant, East Lansing, MI), by a modification of the method of Bonafede et al. (14). Specifically, 100 g of dried dairy product (powdered sweet buttermilk, powdered cultured buttermilk, or lyophilized yogurt) was suspended in 1,000 ml of chloroform:methanol (1:1 v/v) and allowed to stir overnight.

After removal of solid material by Buchner filtration, the extract was subjected to rotary evaporation to remove solvents. The dried extract was resuspended in 50 ml of water and dialyzed against deionized water for 2 days in a cold box. The dialyzed sample was lyophilized and resuspended in chloroform:methanol:water (C:M:W; 30:60:8 v/v) for anion-exchange chromatography. The sample was applied to a 1.5-cm x 30- cm column of DEAD Sephadex A-25 (Sigma Chemical) that was preequilibrated with the C:M:W

solvent. The column was then washed with 175 ml of the C:M:W solvent and 75 ml of methanol. Gangliosides were eluded from the column with 150 ml of 0.4 M ammonium acetate in methanol. Ganglioside-containing fractions were pooled, and methanol was removed by rotary evaporation. The dried ganglioside fraction was resuspended in deionized water and dialyzed against deionized water for 2 days to remove ammonium acetate.

The dialyzed ganglioside fraction was lyophilized and resuspended in minimal deionized water for quantitation, TLC analysis, and hemagglutination studies. LAB gangliosides were extracted in a similar manner except that the LAB cells were ruptured by glass beads in a chilled Bead Beator and cell membranes were recovered after centrifugation of homogenate. The membrane pellet was then extracted with a 20-fold volume of chloroform:methanol (1:1v/v) and processed as described above.

Starter cultures used for the production of yogurt were obtained from Chris Hansen's laboratory (Milwaukee, WI). The starter culture (R-39) contained multiple strains of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. To produce large quantities of LAB for the isolation of bacterial cell wall gangliosides, 0.2 ml of starter culture was inoculated per liter of MRS Broth (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD) and incubated at 39.5°C for 72 h. The resultant culture yielded approximately 7 g of packed wet cells per liter of medium when harvested by centrifugation at 7,650 rpm for 20 min. Approximately 1,400 g of wet bacterial cells were initially propagated and harvested for ganglioside isolation using this method. The starter culture (8803) for buttermilk, which was obtained from Darigold, Seattle, WA,

was also cultivated. The bacterial strains used in this starter culture were proprietary; therefore, specific bacterial identification was not possible.

Ganglioside Quantitation

Thin layer chromatography (TLC) was performed using silica gel 60 TLC precoated plates (glass plates 10 x 10cm Merck). The plates were activated at 100° C and ganglioside preparations and ganglioside and N-acetyl neuraminic acid (NANA) standards were spotted on silica gel G plates and placed in a chamber containing chloroform: methanol:0.25% $CaCL_2$ solution (30:18:4). Plates were developed in the chambers for twice the time it took the solvent to reach the top of the plate. Plates were then removed, dried, sprayed with resorcinol-HCL for visualization, and heated at 110°C for 15 min. Sialic-acid-containing material gave blue spots; organic material lacking sialic acid charred brown. Densitometry of TLC plates was performed with a Shimadzu Dual-Wavelength TLC-Scanner CS930. The chromatograms were obtained by single wavelength zig-zag scanning of each lane at 620 nm (beam dimension: 0.4 X 0.4mm) in the reflection mode (168). The purified preparations yielded blue bands of varying patterns. All types of preparations had a band that migrated with standard GD_3; LAB preparations had only one additional band, whereas the dairy products had six to eight separate ganglioside bands. The yields resulting from this process were small, but the preparations were highly purified and devoid of extraneous contamination.

Source of Erythrocytes

Erythrocytes were obtained from chickens, cows, horses and sheep located at the

Michigan State University, East Lansing, MI farm facilities. Twenty five milliliters of whole blood were drawn from chickens, cows, horses and sheep weekly. Freshly drawn blood was immediately aliquoted to prelabeled chilled test tubes containing an equal volume of Alsever's solution (Sigma Chemical, St. Louis, MO). Test tubes were inverted four times for mixing and placed in an ice bath during transportation. Erythrocytes used for procedures were never older than one week and were stored at 4°C .

Source of Pathogens and Growth Conditions

Strain 80-5022 K88$^+$ *Escherichia coli* , K99$^+$-45 *Escherichia coli* (strain 455133), K99$^+$ *-47 Escherichia coli* (strain 472304P) was provided by the lab of Dr. Robert Holland, Department of Large Animal Clinical Science, Michigan State University or colony forming antigens-I$^+$ *Escherichia coli* (CFA- I$^+$) from the lab of Dr. Raul Hollingsworth, Department of Biochemistry, Michigan State University were utilized. The negative control for K99$^+$ *E. coli* hemagglutination assays was selected to be K88$^+$ *E. coli*. Sheep and horse erythrocytes have receptors for K99$^+$ *E. coli* pili but do not possess receptors for K88$^+$ *E. coli* pili. All tests were performed with 24-h cultures of bacteria (10^8/ml), K99$^+$-45 *E. coli,* K99$^+$-47 *E. coli,* or CFA-I$^+$ *E. coli* grown in Minca broth with IsoVitaleX (BBL/Becton Dickinson, Cockeysville, MD). The cultures were incubated aerobically at 37°C in a shaking water bath prior to use.

Hemagglutination-Inhibition Assay

The crude mixed gangliosides recovered from sweet and cultured buttermilk, yogurt, and lactic acid bacteria (LAB) were tested for their ability to inhibit the pilus-mediated

adhesion of CFA-I$^+$ *E. coli* to cow and chicken erythrocytes, and the K99$^+$ *E. coli* adhesion to horse and sheep erythrocytes. Red blood cells mixed with bovine brain gangliosides (BBG) were used as a positive control. The use of hemagglutination (HA) in the presence or absence of p-mannose and its analogs provides a basic distinction between mannose-sensitive (MS) and mannose-resistant (MR) adhesions and is often used as one form of classification. Enterotoxigenic *E. coli* possesses MR pili, which means they are not inhibited by mannose, and it was therefore included as part of the phosphate buffer.

Pili presence was tested by slide agglutination using CFA-I$^+$, K99$^+$, or K88$^+$ antiserum. One hundred microliters of bacteria (10^8/ml) were mixed with 200 μm of mixed gangliosides; both had been washed in 0.14 M of phosphate-buffered saline (pH 6.8) containing 0.1 M of alpha-methylmannoside (PBSM), and incubated at 4°C for 30 min. Then 100 μl of erythrocytes were added and the mixture was incubated for an additional 60 min at 4°C. Clumping of erythrocytes or hemagglutination was counted in 20 ml of Isoton (BBL/Becton Dickinson, Cockeysville, MD) solution, with an electronic particle counter, Coulter Counter, (Coultronics, Margency, France) tube orifice, 100 μl. All statistical calculations were performed using the Microsoft Excel 2000 statistical package. The percentage of hemagglutination inhibition was calculated using the following standardized formula (116):

$$\frac{RBC_f \text{ in inhibition test} - RBC_f \text{ in 100\% MRHA}}{RBC_f \text{ 0\% MRHA} - RBC_f \text{ in 100 MRHA}} \times 100$$

where:

RBC = Red blood cells

MRHA = Mannose resistant hemagglutination

RBC_f in inhibition test = 100 µl RBC, 200 µl ganglioside, 100 µl bacteria

RBC_f in 100% MRHA = 100 µl RBC, 200 µl buffer, 100 µl bacteria

RBC_f in 0% MRHA = 100 µl RBC, 300 µl buffer

Results

The hemagglutination-inhibition assay is depicted diagrammatically in Figure IV.1. A photograph of the hemagglutination-inhibition assay is shown in Figure IV.2. Images in this dissertation are presented in color. Purified mixed gangliosides extracted from LAB and dairy foods (sweet and cultured buttermilk and yogurt) will inhibit the pilus-mediated hemagglutination of enterotoxigenic *E. coli* to erythrocytes from different livestock species. The percentage of hemagglutination inhibition was calculated using a standardized formula (116). Hemagglutination-inhibition results representing the means of percentage inhibitions at specific concentrations utilizing $K99^+$-47 *E. coli* with sheep and horse erythrocytes are shown in Figures IV.3 through IV.14. As shown in these figures, mixed gangliosides from sweet buttermilk, cultured buttermilk, yogurt and lactic acid bacteria were effective in inhibiting $K99^+$-47 *E. coli* binding to sheep and horse erythrocytes at concentrations ranging from 0.0009 mM to0.38 mM . Hemagglutination was decreased between 0.0% and 100% using these mixed gangliosides. Bovine brain gangliosides (Figures IV.7 and 13) and monosialoganglioside (Figures IV.8 and 14) produced inhibitions ranging from 0.0% to 100% with concentration ranging from 0.0005mM to19.4mM.

As shown in Fig. IV.3 through IV.8, the use of sheep erythrocytes with extracted

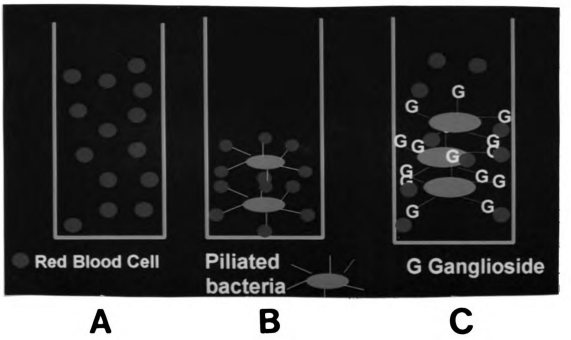


Figure IV.1: Diagrammatic scheme of the hemagglutination-inhibition assay used to determine the beneficial antibinding effect of gangliosides in cultured dairy foods. (a) Red blood cells in buffer showing no binding effect; (b) hemagglutination of red blood cells with piliated bacteria, exhibiting binding; (c) Mixed gangliosides binding to piliated bacteria preventing red blood cells from binding to the piliated bacteria.

Fig. IV.2: Photograph of hemagglutination assay chambers, showing erythrocyte binding to enterotoxigenic *E. coli.* (a) Horse erythrocytes in buffer showing no binding effect; (b) hemagglutination of $K99^+$ *E. coli* with horse erythrocytes, exhibiting binding; (c) horse erythrocytes, $K99^+$ *E. coli,* with mixed gangliosides from cultured dairy foods, showing no binding; (d) horse erythrocytes with $K88^+$ *E. coli* (negative control), showing no binding.

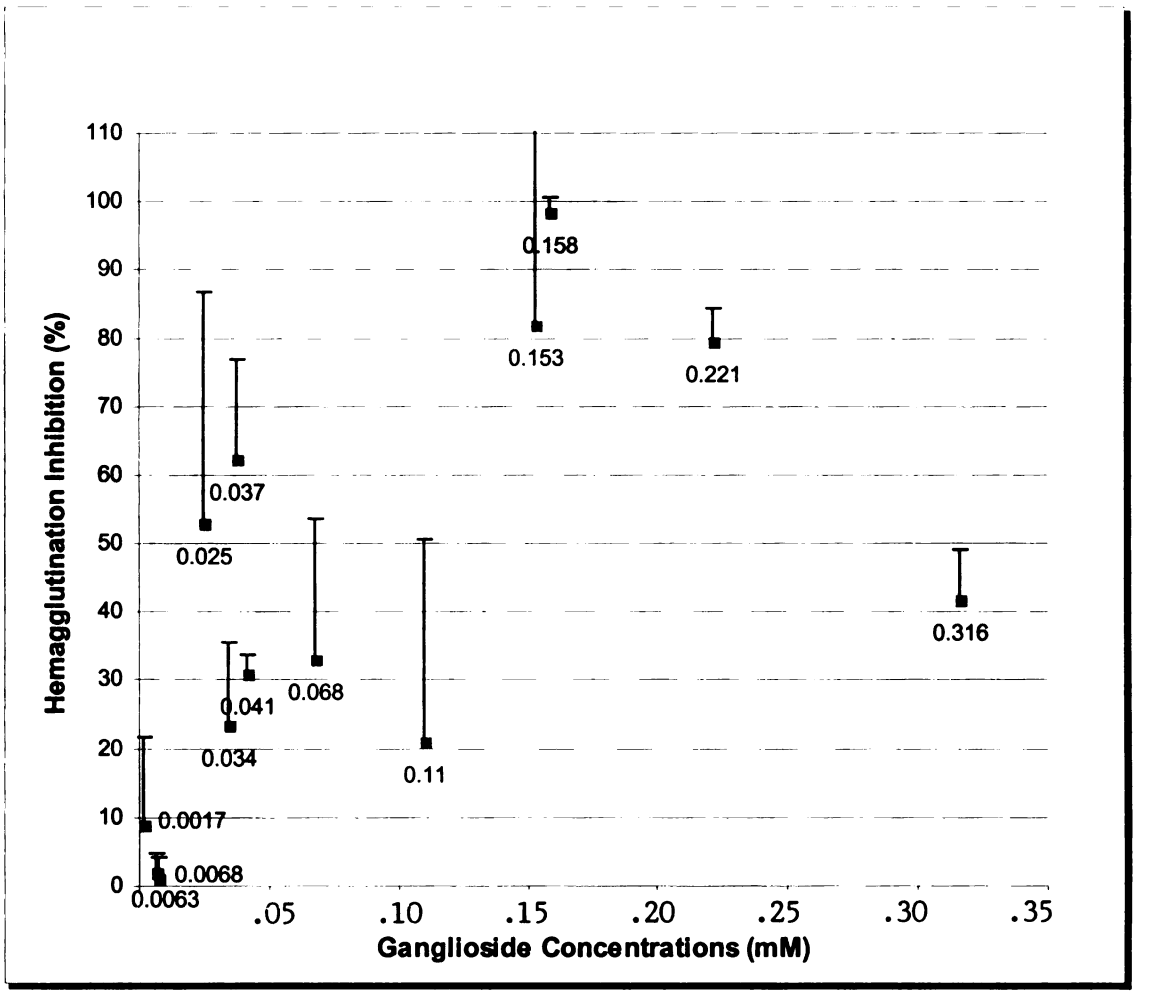


Figure IV.3: Inhibition of sheep erythrocytes with K99$^+$ 47 *Escherichia coli* by competitive binding with sweet butter milk gangliosides. Bacteria (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

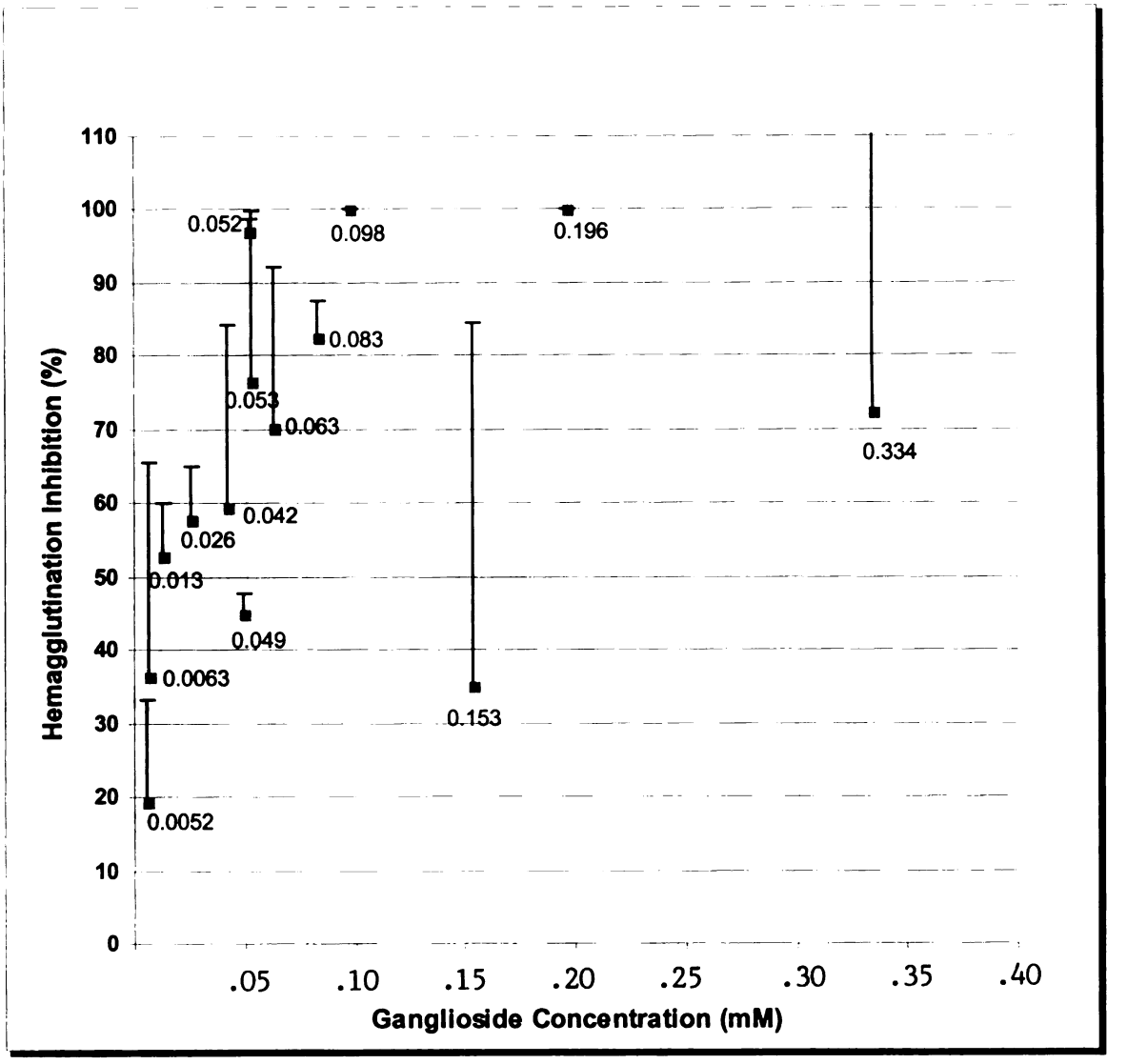


Figure IV.4: Inhibition of sheep erythrocytes with K99$^+$ 47 *Escherichia coli* by competitive binding with cultured butter milk gangliosides. Bacteria (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

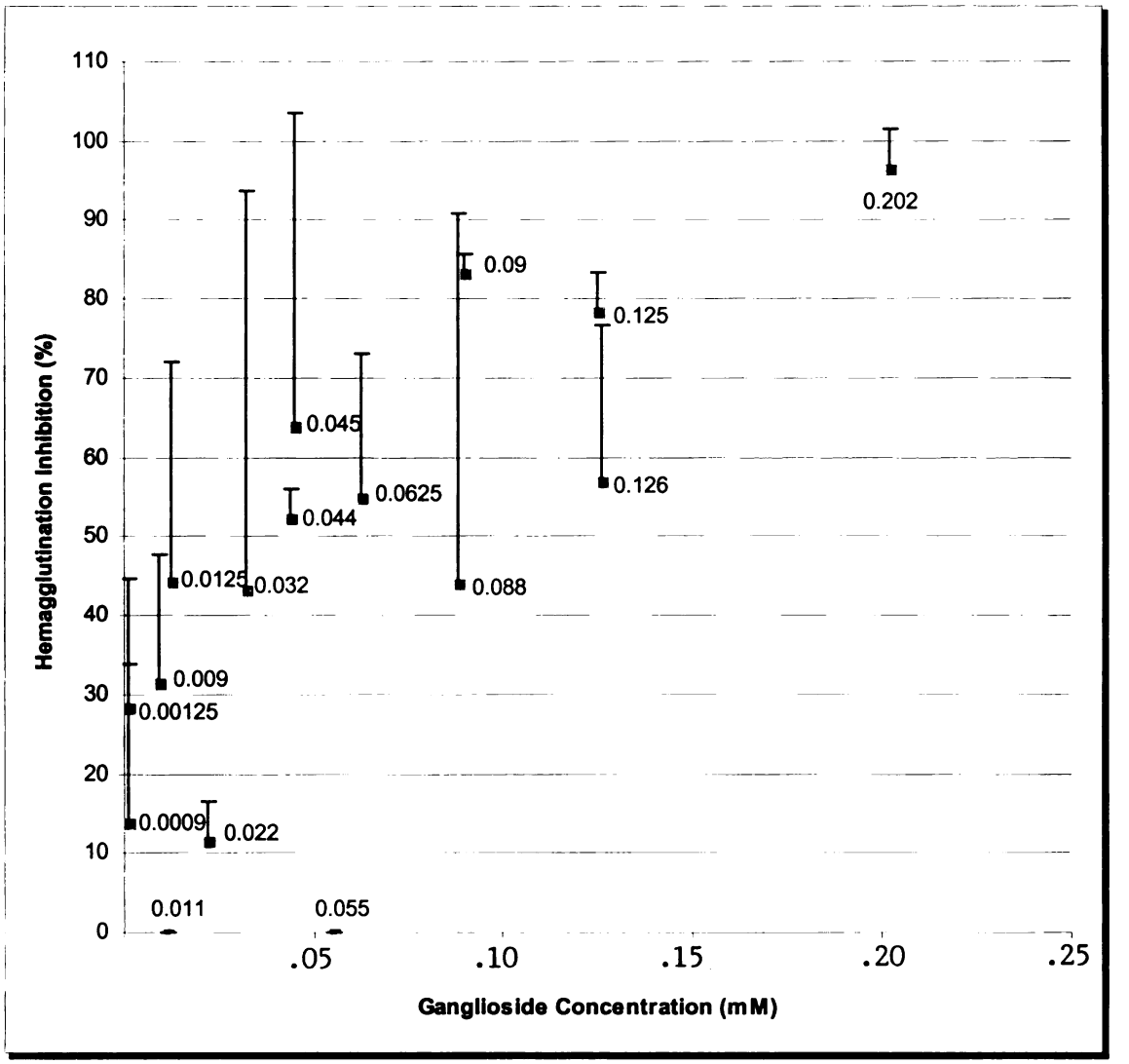


Figure IV.5: Inhibition of sheep erythrocytes with K99$^+$ 47 *Escherichia coli* by competitive binding with yogurt gangliosides. Bacteria (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

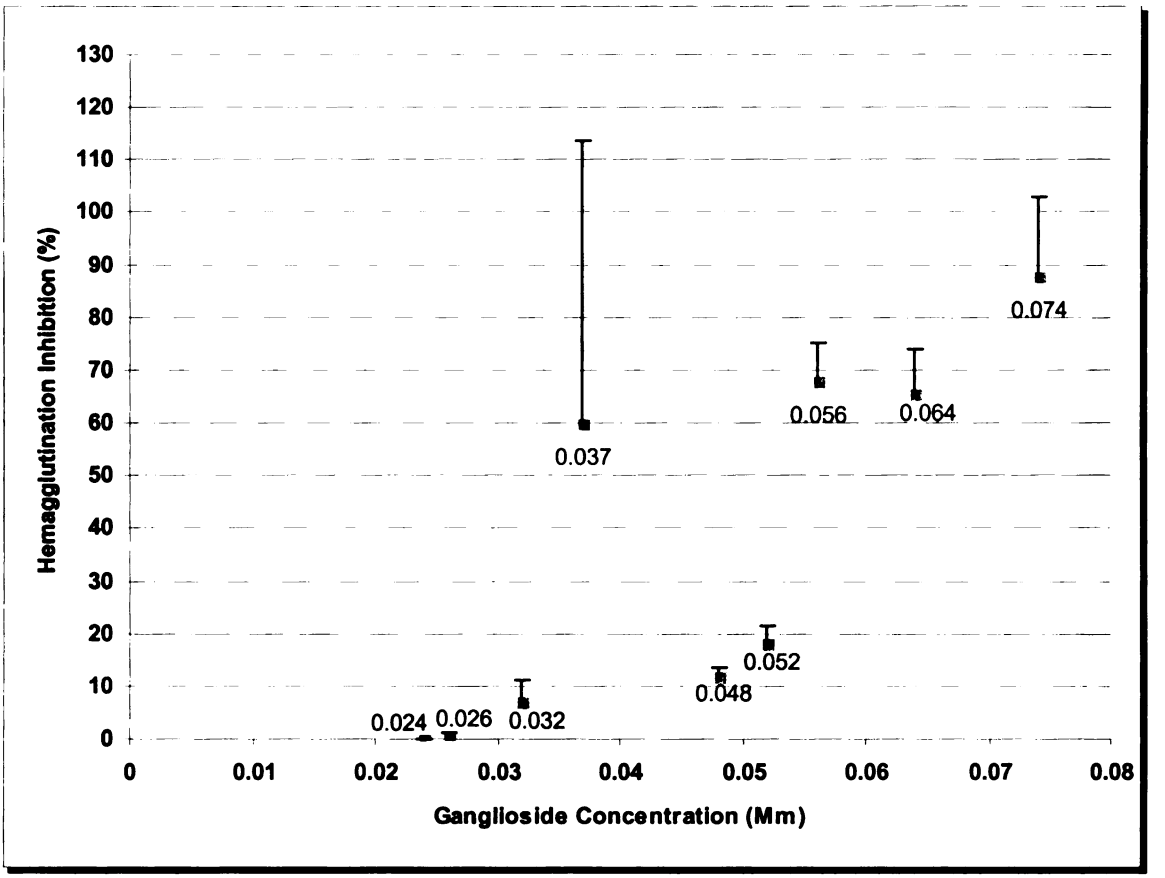


Figure IV.6: Inhibition of sheep erythrocytes with K99$^+$ 47 *Escherichia coli* by competitive binding with lactic acid bacteria gangliosides. Bacteria (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

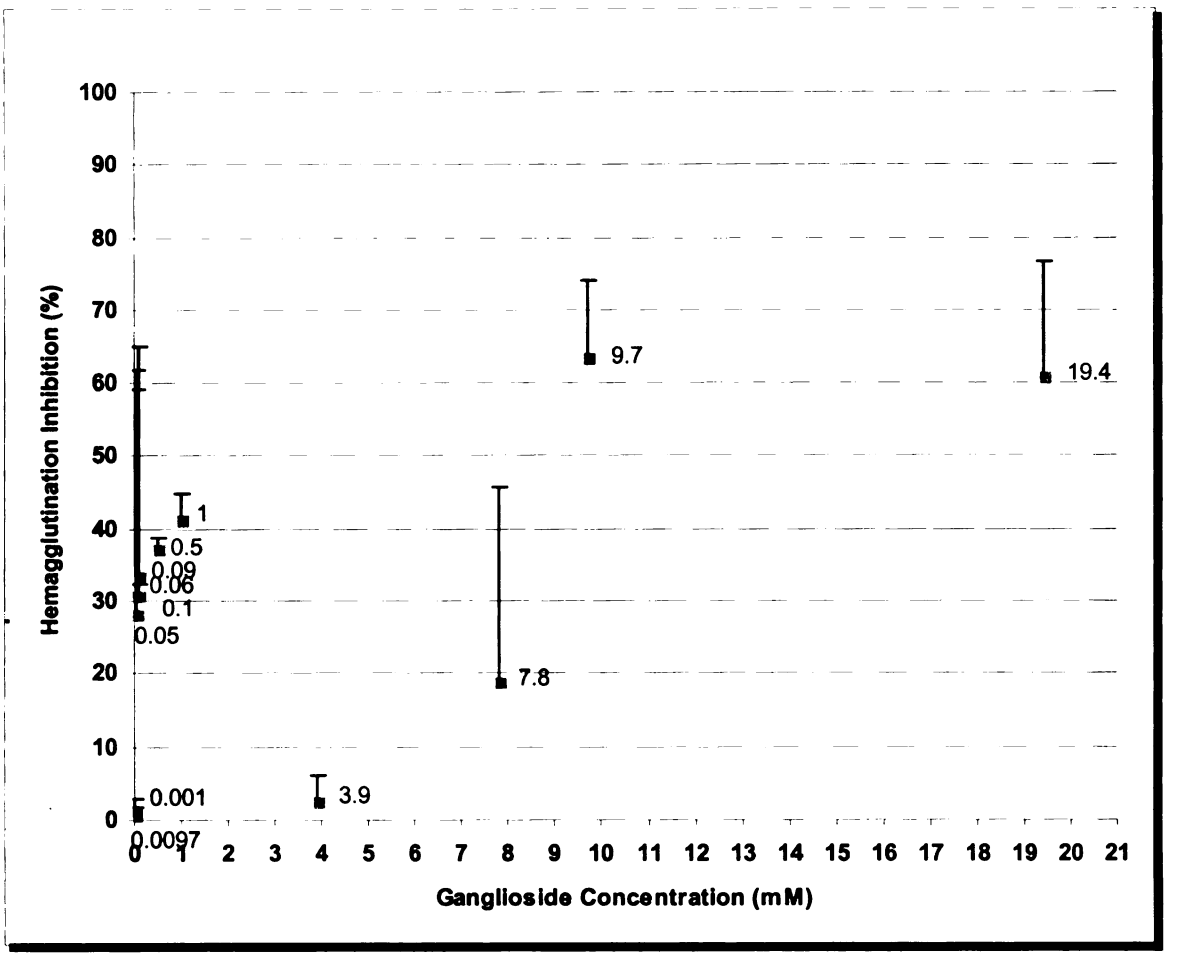


Figure IV.7: Inhibition of sheep erythrocytes with K99$^+$ 47 *Escherichia coli* by competitive binding with bovine brain ganglioside. Bacteria (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

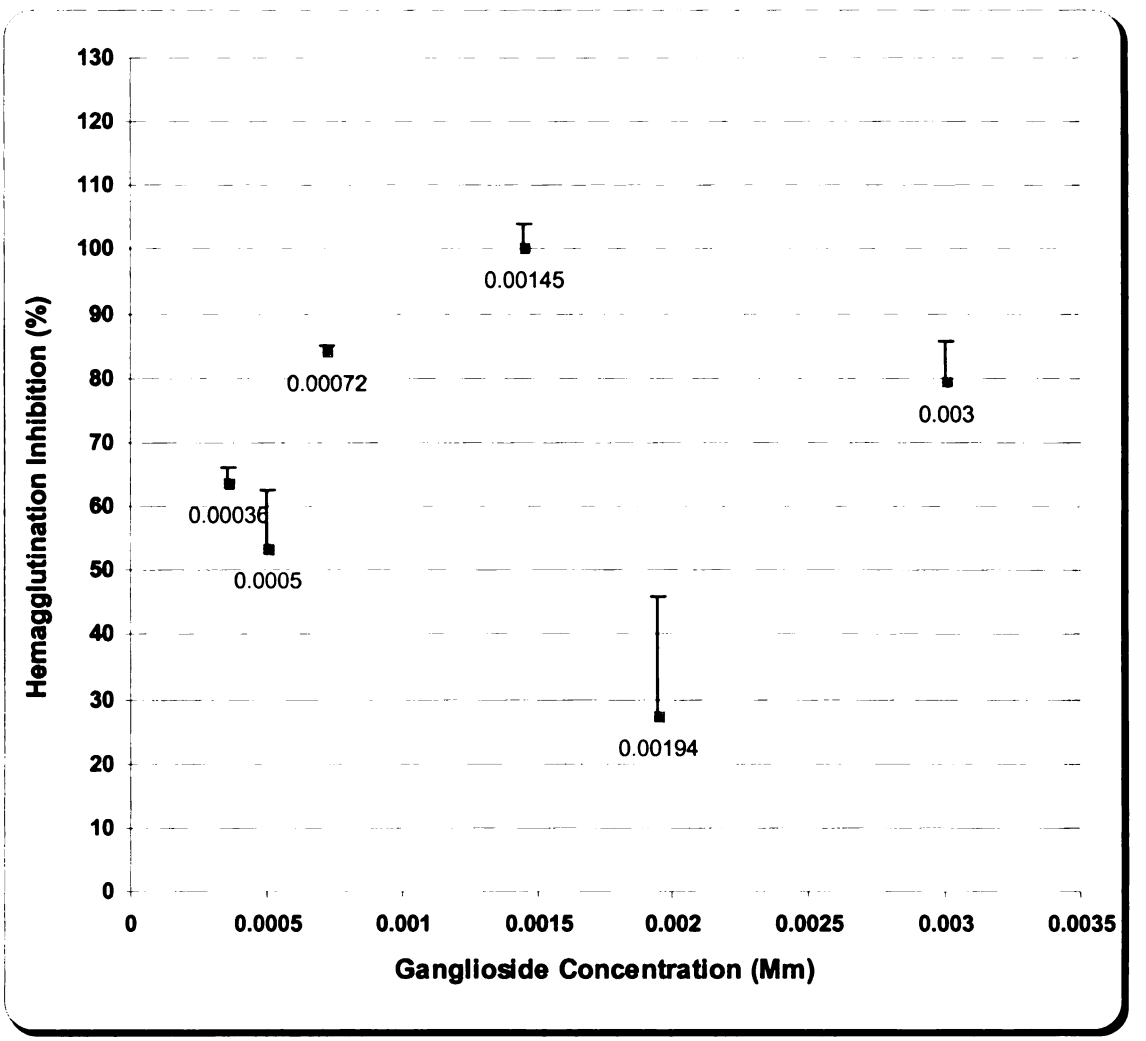


Figure IV.8: Inhibition of sheep erythrocytes with K99$^+$ 47 *Escherichia coli* by competitive binding with monosialoganglioside. Bacteria (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

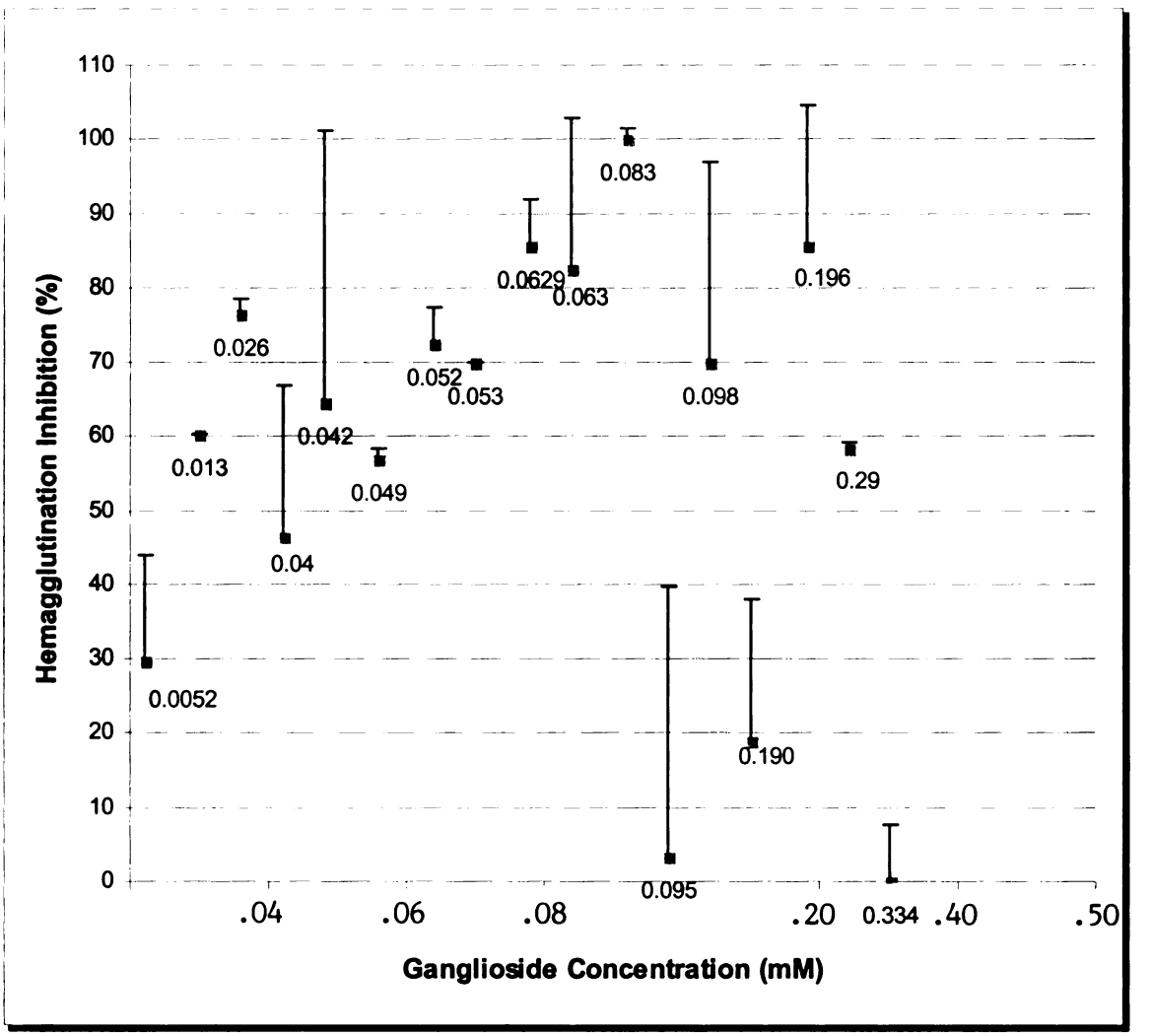


Figure IV.9: Inhibition of equine erythrocytes with K99$^+$ 47 *Escherichia coli* by competitive binding with sweet butter milk gangliosides. Bacteria (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

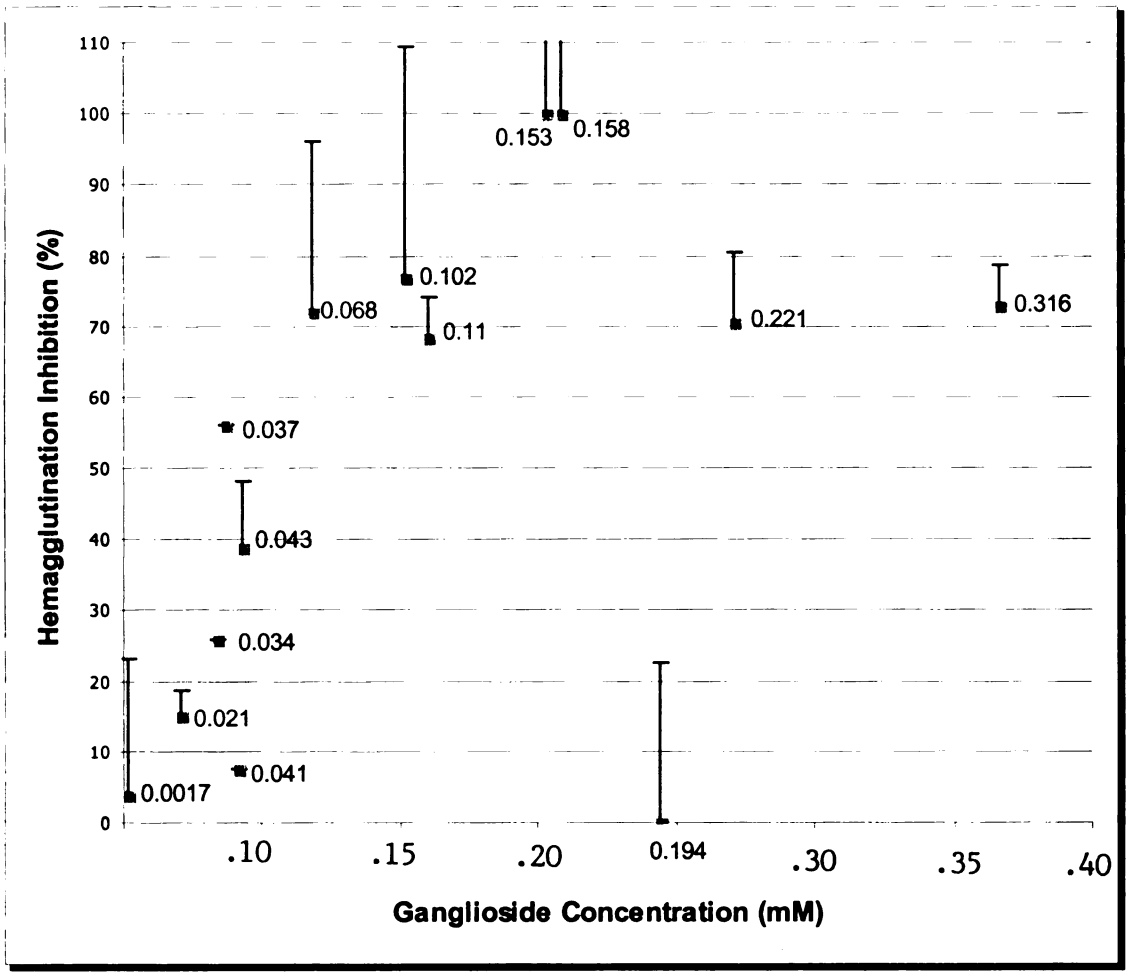


Figure IV.10: Inhibition of equine erythrocytes with K99$^+$ 47 *Escherichia coli* by competitive binding with cultured butter milk gangliosides. Bacteria (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

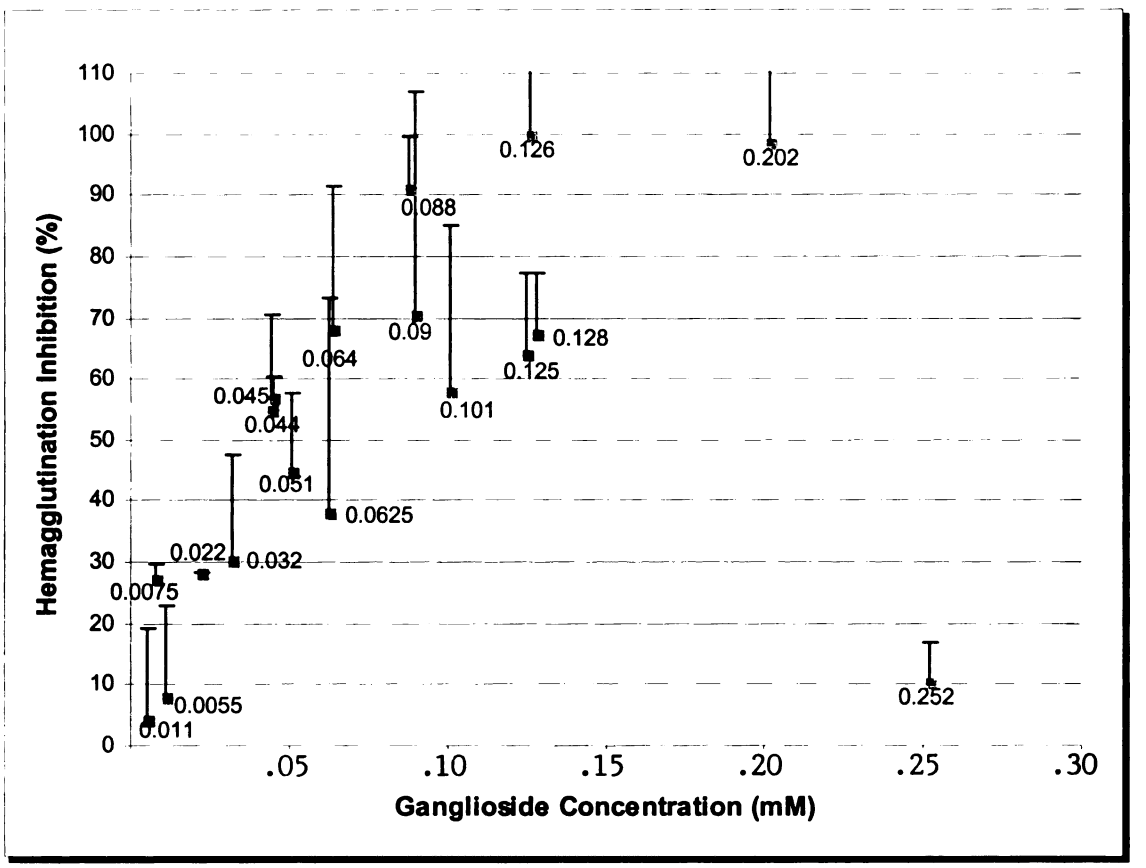


Figure IV.11: Inhibition of equine erythrocytes with K99$^+$ 47 *Escherichia coli* by competitive binding with yogurt gangliosides. Bacteria (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

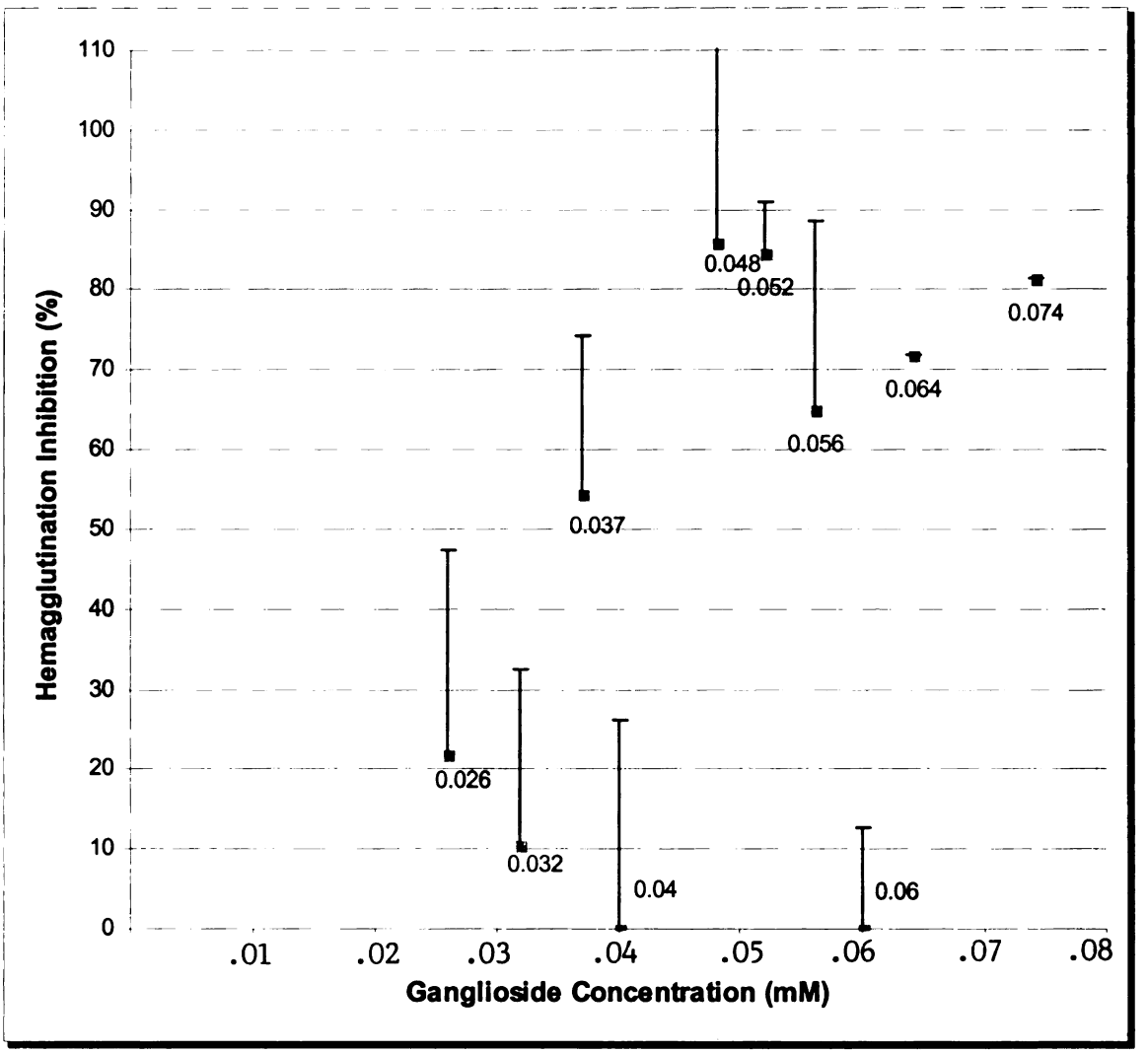


Figure IV.12: Inhibition of equine erythrocytes with K99$^+$ 47 *Escherichia coli* by competitive binding with lactic acid bacteria gangliosides. Bacteria (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

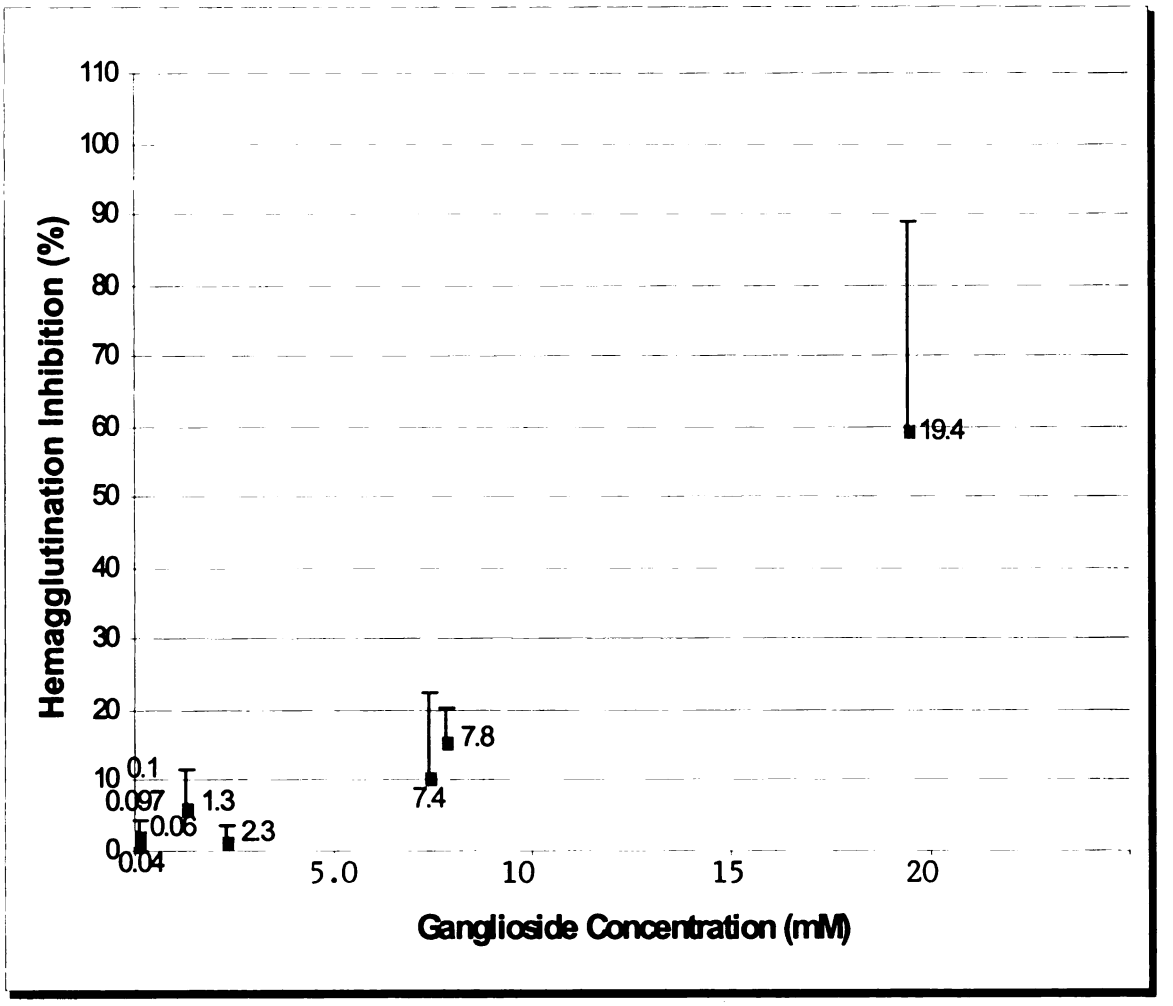


Figure IV.13: Inhibition of equine erythrocytes with K99$^+$ 47 *Escherichia coli* by competitive binding with bovine brain gangliosides. Bacteria (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

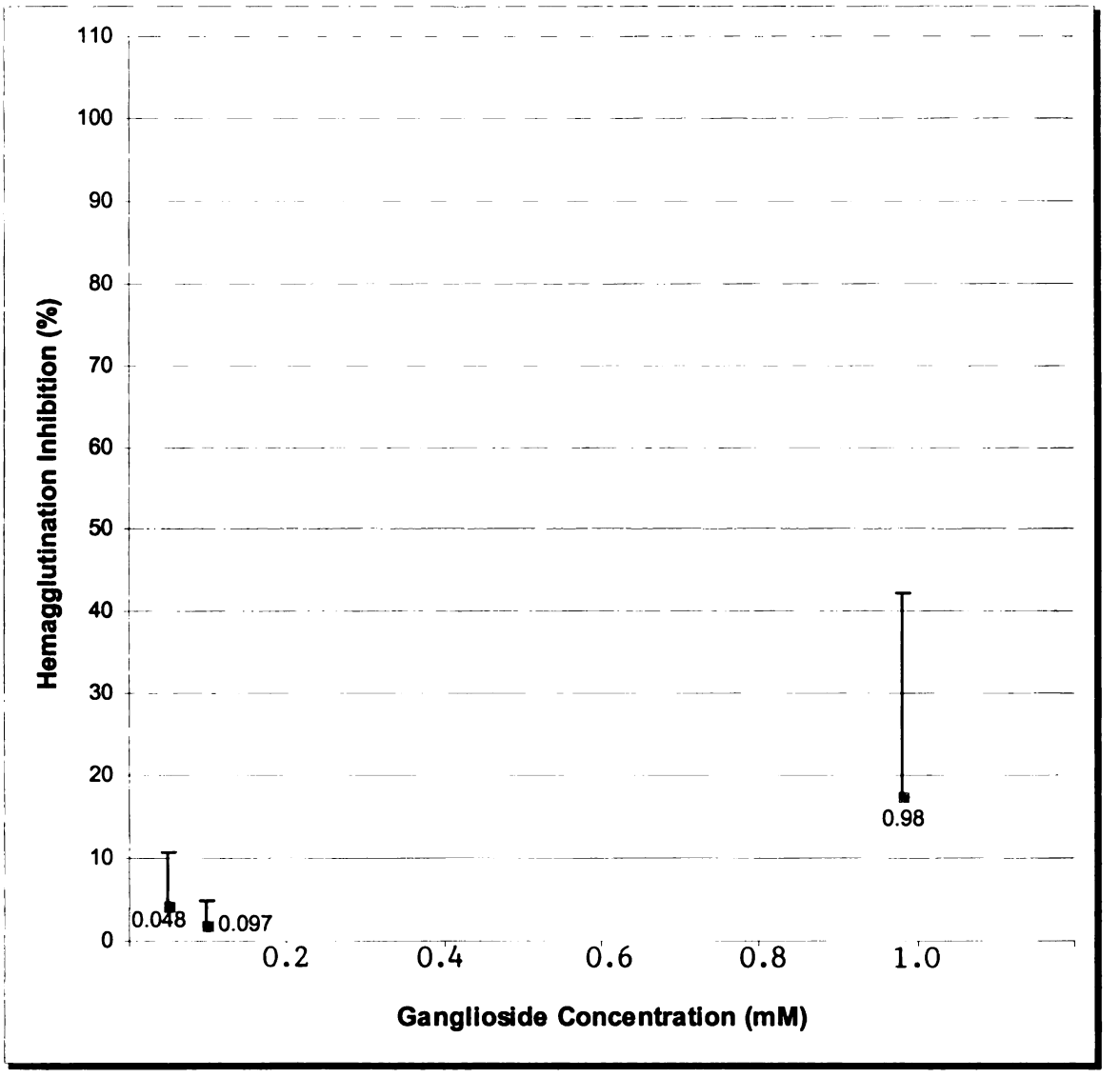


Figure IV.14: Inhibition of equine erythrocytes with K99$^+$ 47 *Escherichia coli* by competitive binding with monosialoganglioside-3. Bacteria (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

mixed gangliosides produced the following ranges of hemagglutination-inhibition percentages, standard deviations and standard error of the mean. Sweet buttermilk ganglioside concentrations (Fig. IV.3) in the range of 0.0017mM to 0.316mM produced inhibitions ranging from 2% to 98.5%; standard deviations (Sd) ranging from 2.08 standard deviation units (SdU) to 33.9SdU and standard error of the mean (SE_m) ranging from 1.49SE_m units (SE_mU) to 23.99SE_mU. Additionally, when concentrations of 0.0017mM and 0.316mM sweet buttermilk gangliosides were compared, the percent inhibitions were determined to be significant at a confidence level of 0.01 utilizing the t-Test. Cultured buttermilk ganglioside concentrations (Fig. IV.4) in the range of 0.0052 mM to 0.334 mM produced inhibitions ranging from 19.6% to 100%; standard deviations(Sd) ranging from 1.73SdU to 49.49SdU and standard error of the mean (SE_m) ranging from 0.99 Se_mU to 34.99 Se_mU. When concentrations of 0.0052mM and 0.33mM cultured buttermilk gangliosides were compared, the percent inhibitions were determined to be significant at a confidence level of 0.05 utilizing the t-Test. Yogurt ganglioside concentrations (Fig. IV.5) in the range of 0.0009 mM to 0.202 mM produced inhibitions ranging from 0% to 96%; Sd ranging from 2.12SdU to 50.2SdU and SE_m ranging from 1.49SE_mU to 35.35Se_mU. Additionally, when concentrations of 0.0009mM and 0.02mM gangliosides were compared, the percent inhibitions were determined to be significant at a confidence level of 0.05 utilizing the t-Test. Lactic acid bacteria ganglioside concentrations (Fig. IV.6) in the range of 0.024 mM to 0.074mM produced inhibitions ranging from 0% to 88%; Sd ranging from 0.70SdU to 53.74SdU and SE_m ranging from 0.49Se_mU to 37.47Se_mU. When concentrations of 0.024mM and 0.074mM gangliosides were compared, the percent inhibitions were determined to be significant at a confidence

level of 0.01 utilizing the t-Test. Bovine brain gangliosides concentrations (Fig. IV.7) in the range of 0.0097mM to19.4mM produced inhibitions ranging from 0% to 63.5%; Sd ranging from 0.0SdU to 26.87SdU and SE_m ranging from 0.49Se_mU to 18.99Se_mU. Additionally, when concentrations of 0.0097mM and 19.4mM gangliosides were compared, the percent inhibitions were determined to be significant at a confidence level of 0.01 utilizing the t-Test. It is important to note that bovine brain ganglioside concentrations required to produce effective inhibitions was increased by a factor of 10, as compared to the mixed gangliosides extracted from sweet and cultured butter milk and lactic acid bacteria. This observation held true for the different gangliosides used, the various pathogens inhibited, and the types of erythrocytes tested. An additional assay, using monosialoganglioside-3 (GM_3) (Sigma Chemical, St Louis, MO), which has been reported to be the $K99^+$ receptor site (84), was also conducted (Fig. IV.8). Monosialoganglioside-3 (GM_3) concentrations ranging from 0.0005mM to 0.003 mM produced inhibitions ranging from 27% to 100%; Sd ranging from 0.70SdU to 18.38SdU and SE_m ranging from 0.50SE_mU to 12.99Se_mU. Further, when monosialoganglioside-3 (GM_3) (Fig. IV.8) and bovine brain gangliosides (BBG) (Fig. IV.7) were compared at the concentration of 0.001mM utilizing sheep erythrocytes, monosialoganglioside-3 (GM_3) demonstrated a greater ability to inhibit agglutination. These findings were consistent using either horse or sheep erythrocytes.

Depicted in Figures IV.9 through IV.13 are the results of equine erythrocytes binding with $K99^+$ *E. coli* and incubated with extracted mixed gangliosides, producing the following ranges of hemagglutination-inhibition percentages, standard deviations and

standard errors of the mean. Sweet buttermilk ganglioside concentrations (Fig. IV.9) in the range of 0.0052 mM to 0.38 mM produced inhibitions ranging from 0% to 100%; Sd ranging from 0.70SdU to 36.76SdU and SE_m ranging from 0.49Se_mU to 25.99SE_mU. Additionally, when concentrations of 0.0052mM and 0.380mM gangliosides were compared, the differences between percent inhibitions were determined to be significant at a confidence level of 0.05 utilizing the t-Test. Cultured butter milk ganglioside concentrations (Fig. IV.10) in the range of 0.0017 mM to 0.316 mM produced inhibitions ranging from 4% to 100%; Sd ranging from 0.0SdU to 32.52SdU and SE_m ranging from 0.0SE_mU to 22.99Se_mU. When concentrations of 0.0017mM and 0.316mM gangliosides were compared, the differences between percent inhibitions were determined to be significant at a confidence level of 0.01 utilizing the t-Test. Yogurt ganglioside concentrations (Fig. IV.11) in the range of 0.0055mM to 0.252mM produced inhibitions ranging from 4% to 100%; Sd ranging from 2.12SdU to 36.59SdU and SE_m ranging from 1.49SE_mU to 25.87Se_mU. Additionally, when concentrations of 0.0055mM and 0.202mM yogurt gangliosides were compared, the percent inhibition of the larger concentration was determined to be significantly greater at a confidence level of 0.01 utilizing the t-Test. Lactic acid bacteria ganglioside concentrations (Fig. IV.12) in the range of 0.04 mM to 0.074 mM produced inhibitions ranging from 0% to 86%; Sd ranging from 6.36SdU to 40.30SdU and SE_m ranging from 4.49SE_mU to 28.49Se_mU. Additionally, when concentrations of 0.026mM and 0.074mM lactic acid bacteria gangliosides were compared, the differences between percent inhibitions were determined to be significant at a confidence level of 0.01 utilizing the t-Test. Bovine brain ganglioside concentrations (Fig. IV.13) in the range of 0.04mM to 19.4mM produced inhibitions ranging from 0% to

60%; Sd ranging from 2.82SdU to 29.14SdU and SE_m ranging from 1.99SE_mU to 20.60Se_mU. Additionally, when concentrations of 0.04mM and 19.4mM gangliosides were compared, the percent inhibition of the larger concentration was determined not to be significantly greater at a confidence level of 0.05 utilizing the t-Test.The hemagglutination assay using GM_3 (Sigma Chemical, St. Louis, MO) (Fig. IV.14) in the range of 0.48 mM to 0.98 mM produced inhibitions ranging from 2% to 17.5%; Sd ranging from 2.82SdU to 24.74SdU and SE_m ranging from 1.99SE_mU to 17.49Se_mU. Additionally, when concentrations of 0.48mM and 0.98mM GM_3 (Sigma Chemical, St. Louis, MO) were compared, the percent inhibition of the larger concentration was determined not to be significantly greater at a confidence level of 0.05 utilizing the t-Test. Testing the percent inhibitions of 0.10mM bovine brain ganglioside (Fig. IV.13) with 0.98mM monosialoganglioside-3 (Fig. 4.14) it was determined that they were not significantly different at a confidence level of 0.05 utilizing the t-Test. The hemagglutination inhibition percentage of lactic acid bacteria ganglioside at a concentration of 0.074mM was evaluated using sheep erythrocytes and equine erythrocytes and it was determined that they were not significantly different at a confidence level of 0.05 utilizing the t-Test.

Figures IV.15 through IV.18 demonstrate the inability of $K88^+$ *E. coli* to inhibit hemagglutination with equine erythrocytes due to the absence of a $K88^+$ *E. coli* receptor on the surface of equine erythrocytes. Fig. IV.15 illustrates the use of sweet buttermilk gangliosides, Fig. IV.16 depicts the use of cultured buttermilk gangliosides, Fig. IV.17 illustrates the use of yogurt gangliosides, and the use of bovine brain ganglioside is illustrated in Fig. IV.18. The mean percent inhibition for $K88^+$ *E. coli* with sweet

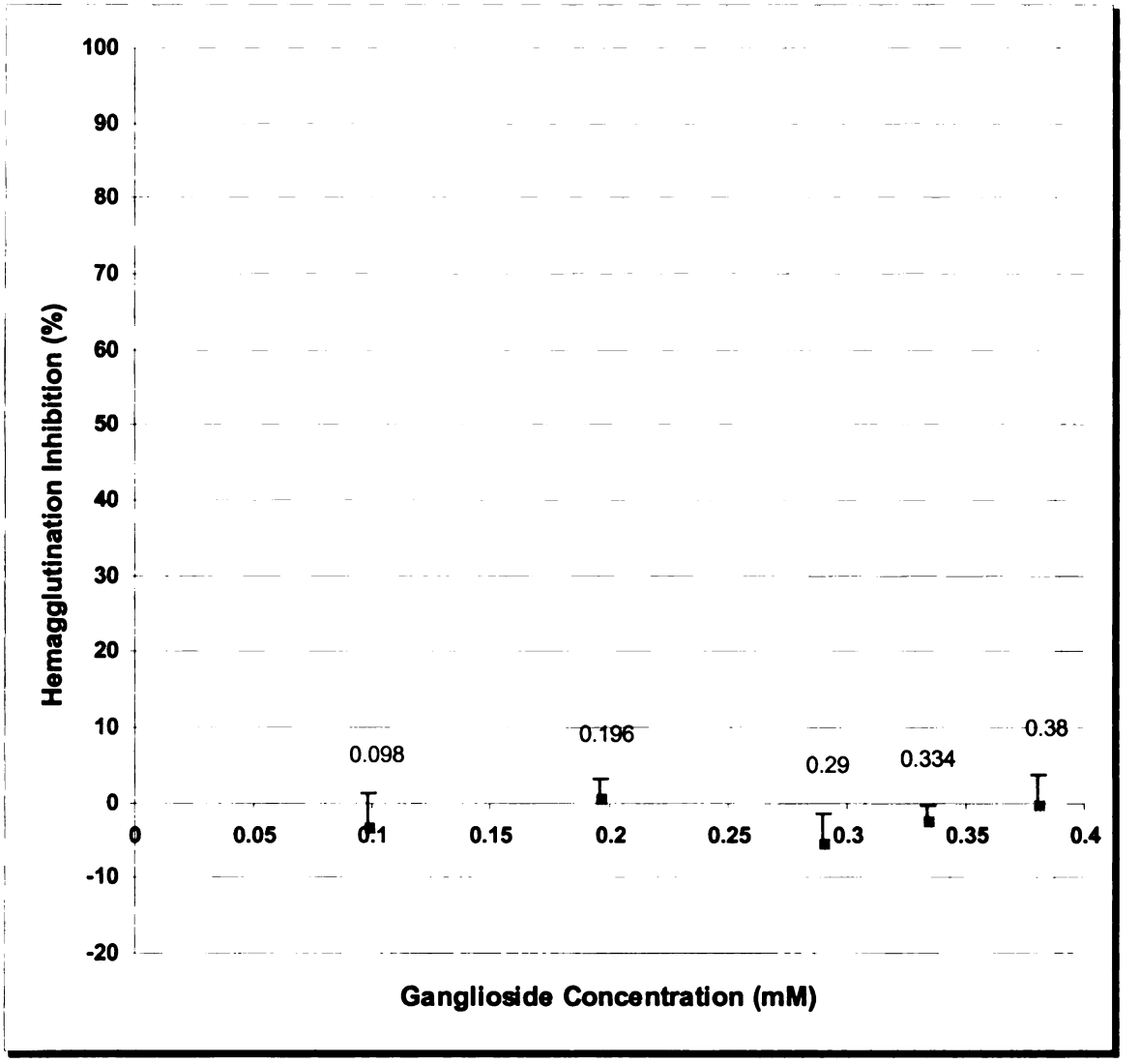


Figure IV.15: Inhibition of equine erythrocytes with K88$^+$ *Escherichia coli* by competitive binding with sweet butter milk gangliosides. Bacteria (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

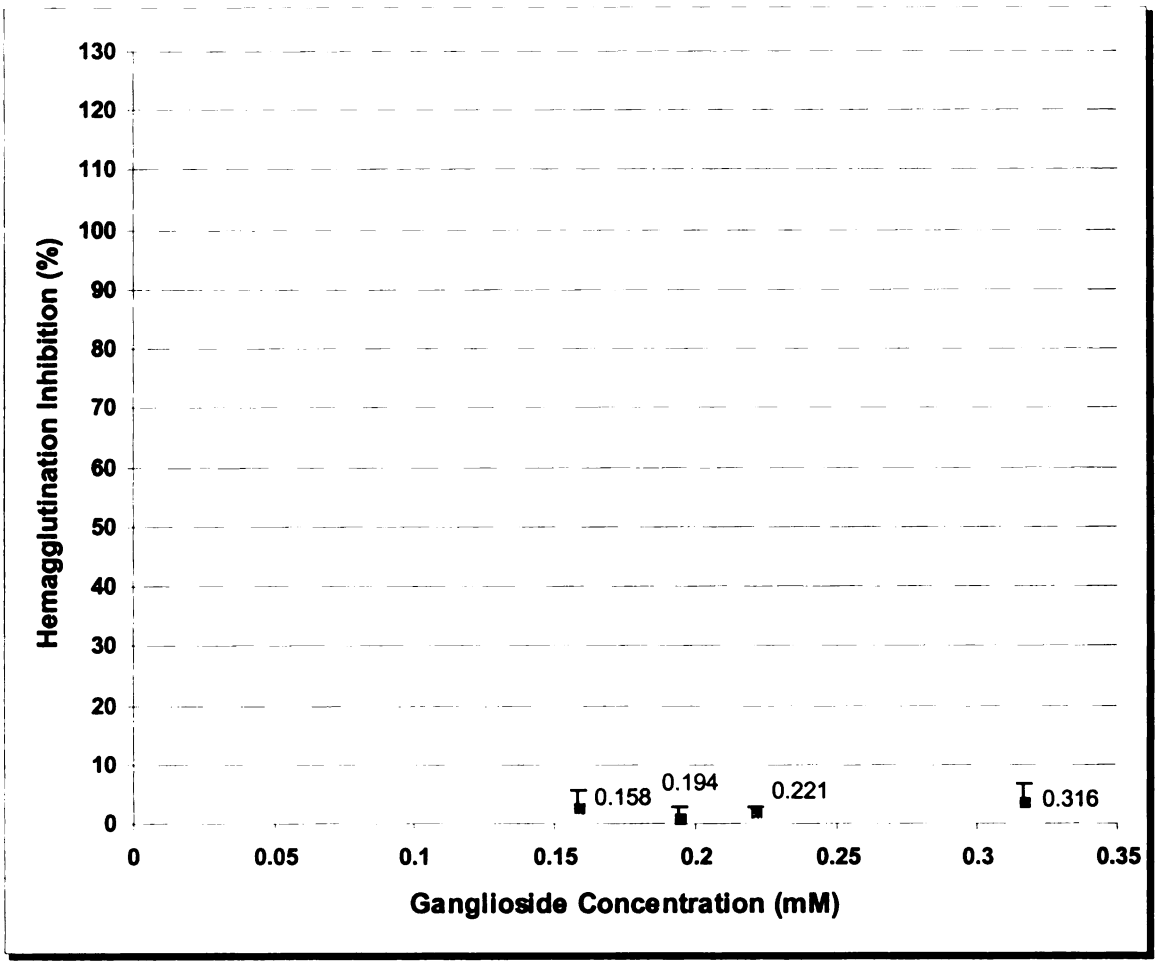


Figure IV.16: Inhibition of equine erythrocytes with K88$^+$ *Escherichia coli* by competitive binding with cultured butter milk gangliosides. Bacteria (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

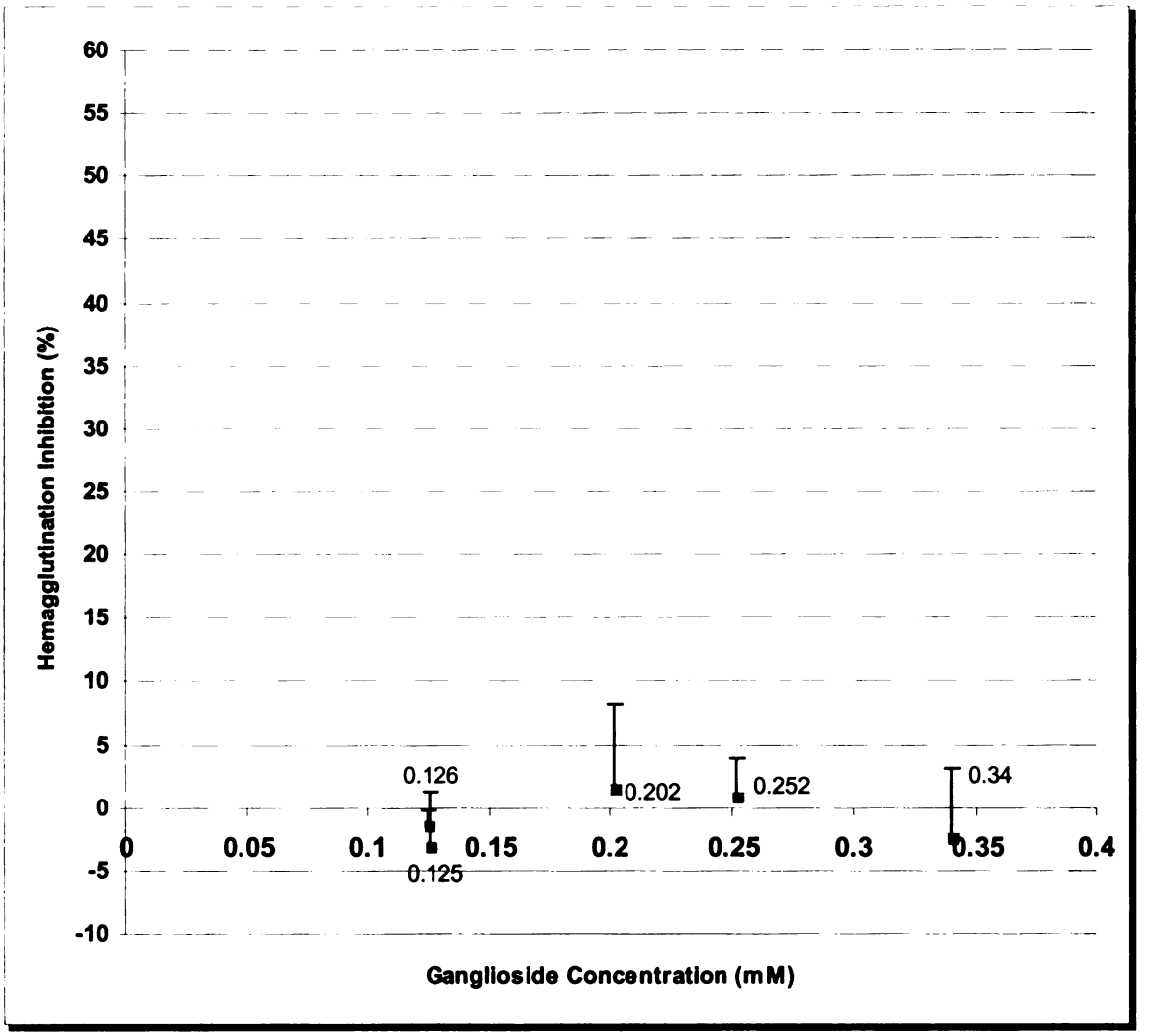


Figure IV.17: Inhibition of equine erythrocytes with K88$^+$ *Escherichia coli* by competitive binding with yogurt gangliosides. Bacteria (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

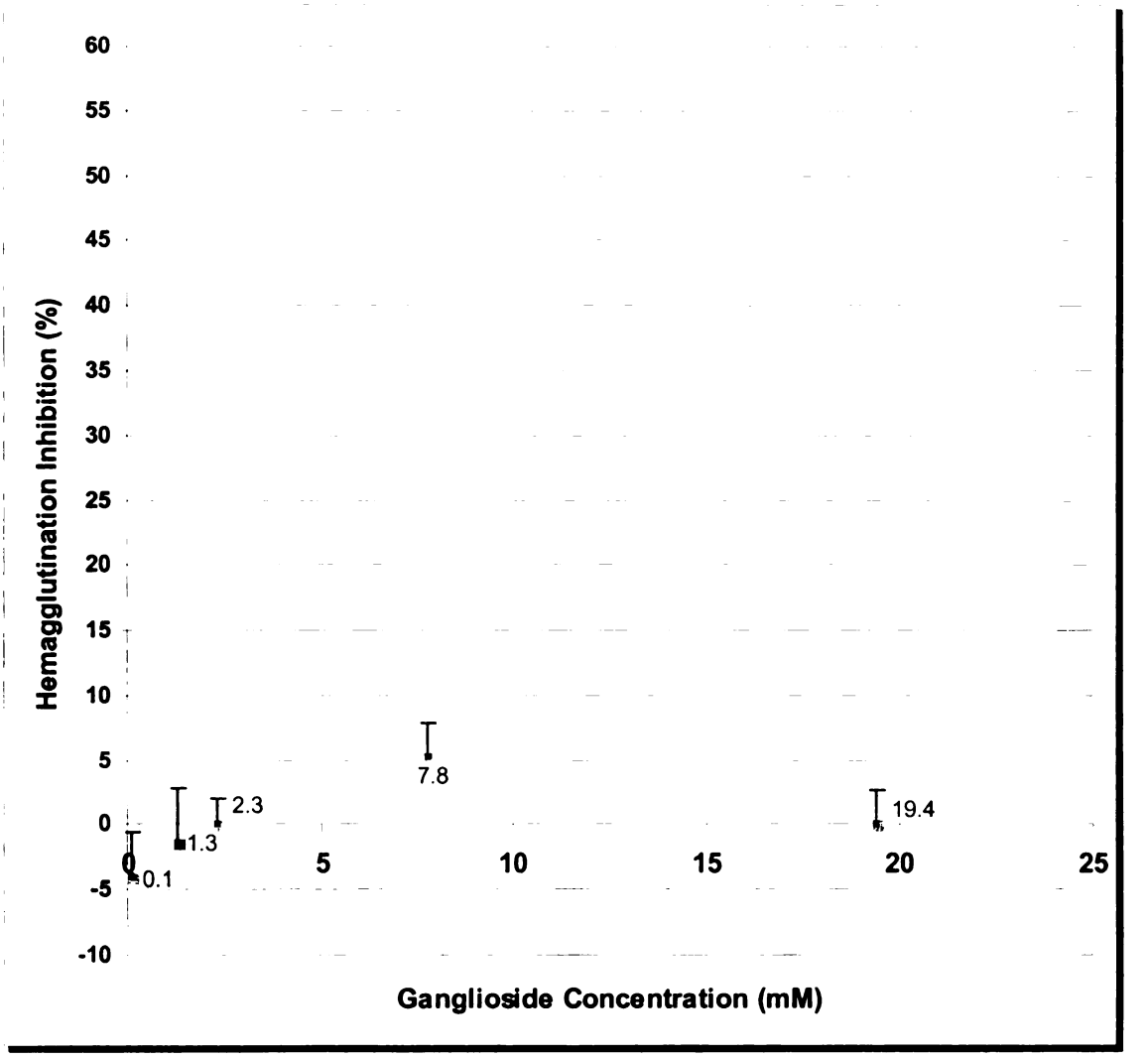


Figure IV.18: Inhibition of equine erythrocytes with K88^{+} *Escherichia coli* by competitive binding with bovine brain gangliosides. Bacteria (10^{8}/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

buttermilk ganglioside, cultured buttermilk gangliosides yogurt gangliosides and bovine brain ganglioside was -1%. As demonstrated in Fig. IV.19 through IV.26, mixed gangliosides from sweet buttermilk, cultured butter milk, and lactic acid bacteria were effective in inhibiting the pilus-mediated agglutination of colonization factor antigen-I (CFA-I) to chicken and bovine erythrocytes at concentrations ranging from 0.016mM to 0.176 mM. Hemagglutination was inhibited from 32% to 98.5% using these mixed gangliosides. Depicted in Figures IV.19 through IV.22 are the results of CFA^{+}-I and chicken erythrocytes which produced the following ranges of hemagglutination-inhibition percentages, standard deviations and standard error of the mean. Sweet buttermilk ganglioside concentrations (Fig. IV.19) in the range of 0.066 mM to 0.136mM produced inhibitions ranging from 44% to 98%; Sd ranging from 3.53SdU to 14.46SdU and SE_m ranging from 2.49SE_mU to 8.35Se_mU. Additionally, when concentrations of 0.066mM and 0.136mM sweet buttermilk gangliosides were compared the differences between percent inhibitions were determined to be significant at a confidence level of 0.01 utilizing the t-Test. Cultured buttermilk ganglioside concentrations (Fig. IV.20) in the range of 0.079mM to 0.18mM produced inhibitions ranging from 46% to 49%; Sd ranging from 4.24SdU to 6.36SdU and Se_m ranging from 2.94SE_mU to 4.49SE_mU. Comparing concentrations of 0.079mM to 0.18mM cultured buttermilk gangliosides, the differences between percent inhibitions were determined to be significant at a confidence level of 0.01 utilizing the t-Test. Mixed yogurt ganglioside concentrations (Fig. IV.21) in the range of 0.016mM to 0.176 mM produced inhibitions ranging from 49% to 81%; Sd ranging from 0.70SdU to 5.74SdU and SE_m ranging from 0.0SE_mU to 2.87Se_mU.

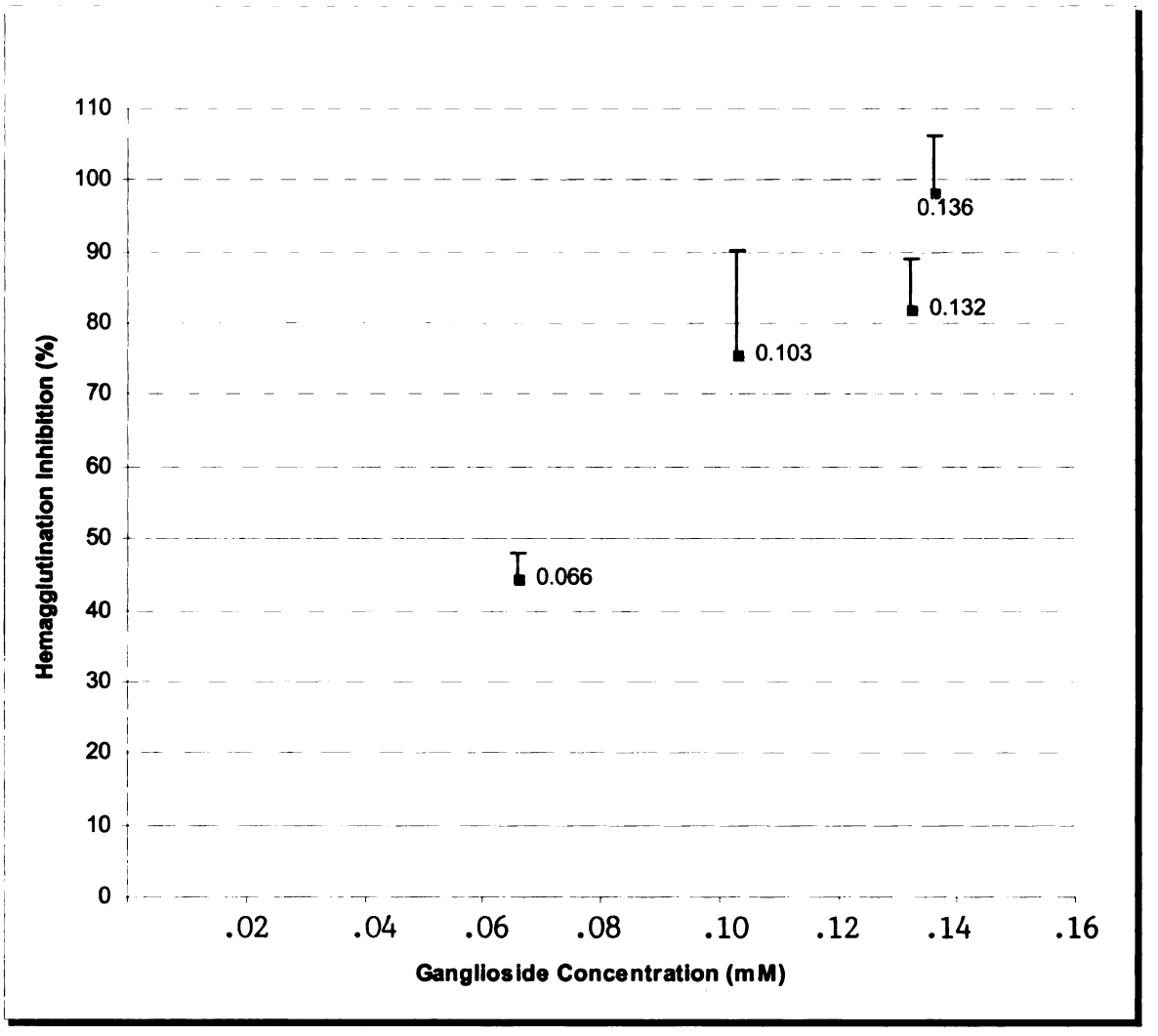


Figure IV.19: Inhibition of chicken erythrocytes with colonization factor antigen-I (CFA-I) by competitive binding with sweet butter milk gangliosides. CFA-I (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

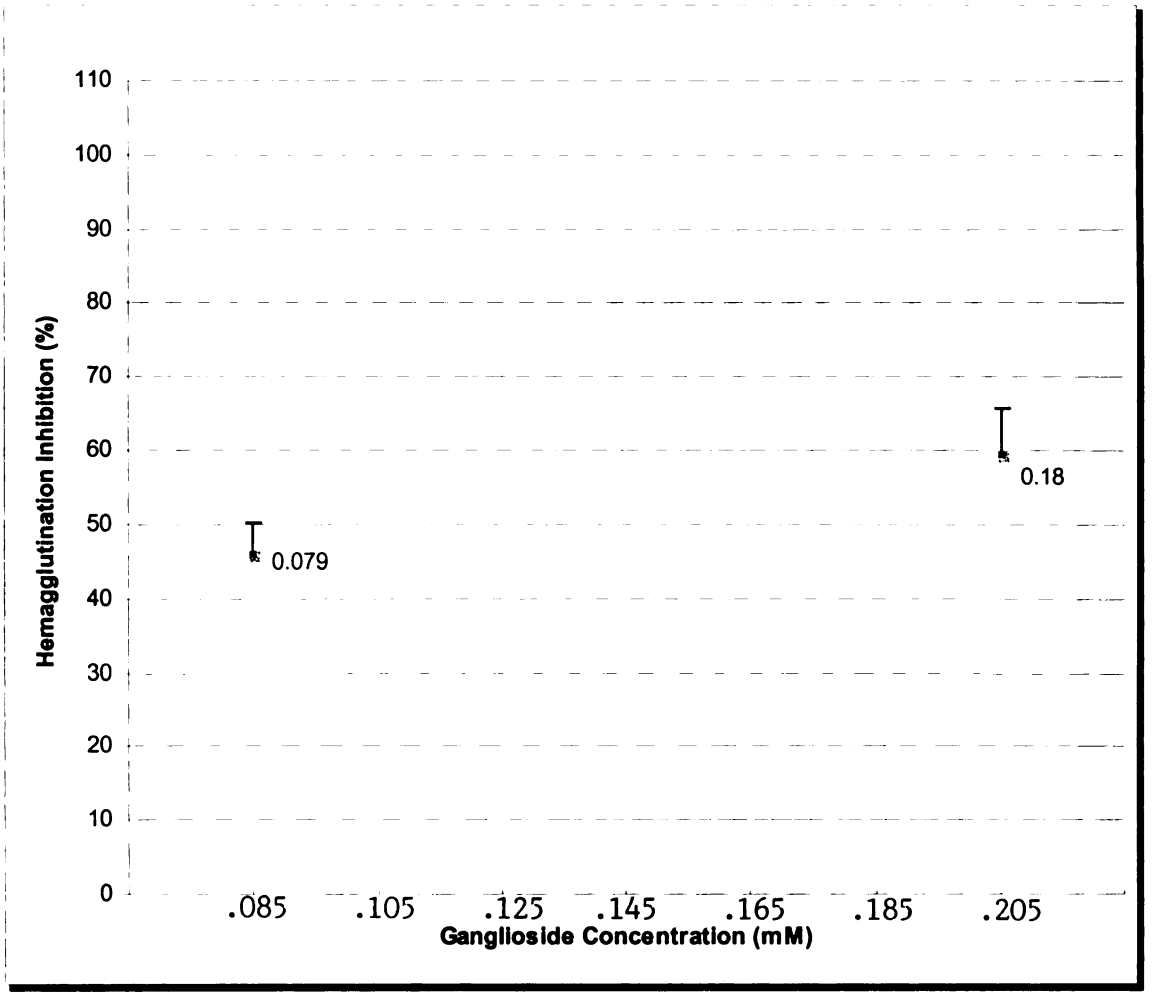


Figure IV.20: Inhibition of chicken erythrocytes with colonization factor antigen-I (CFA-I) by competitive binding with cultured butter milk gangliosides. CFA-I (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

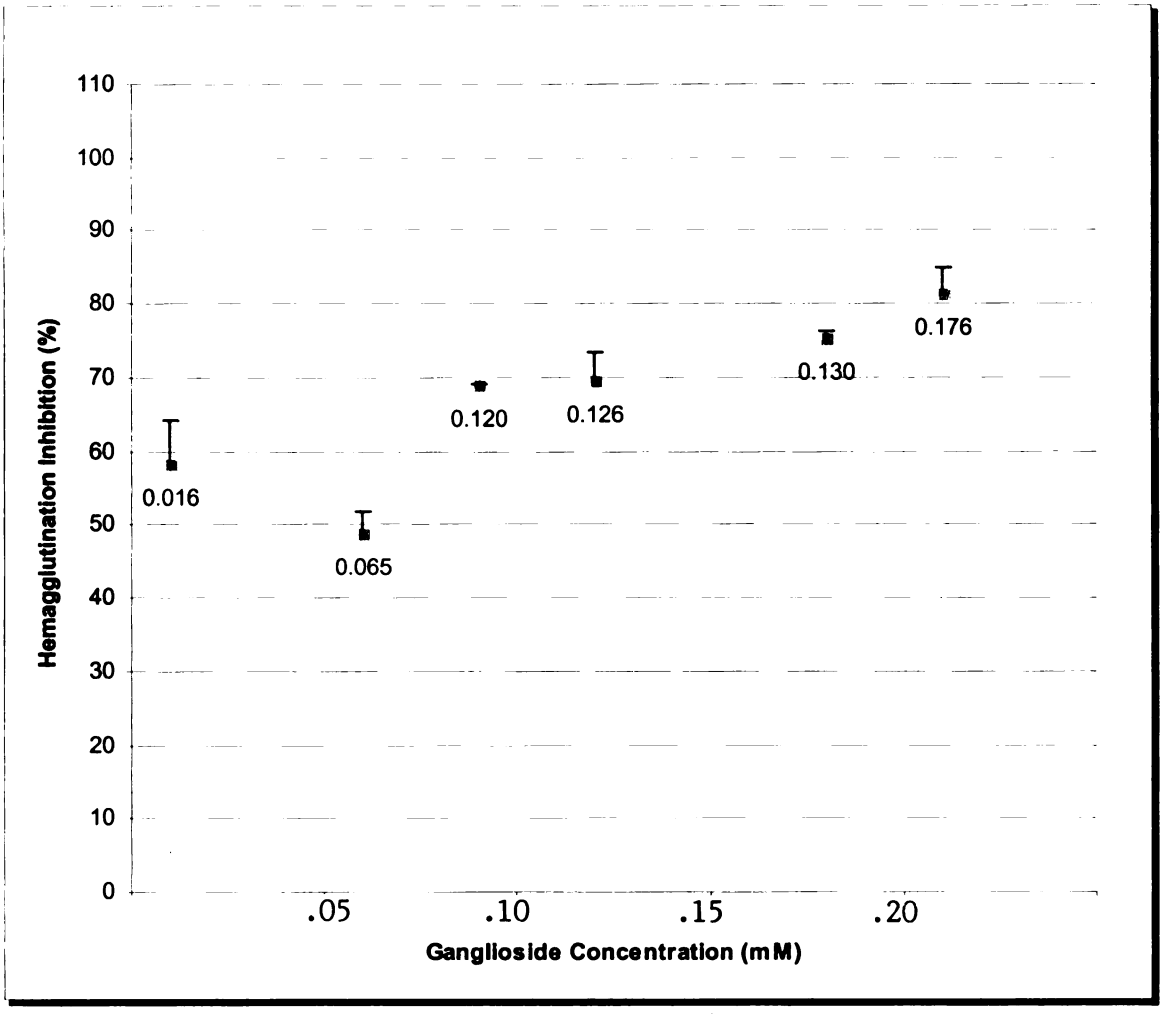


Figure IV.21: Inhibition of chicken erythrocytes with colonization factor antigen-I (CFA-I) by competitive binding with yogurt gangliosides. CFA-I (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

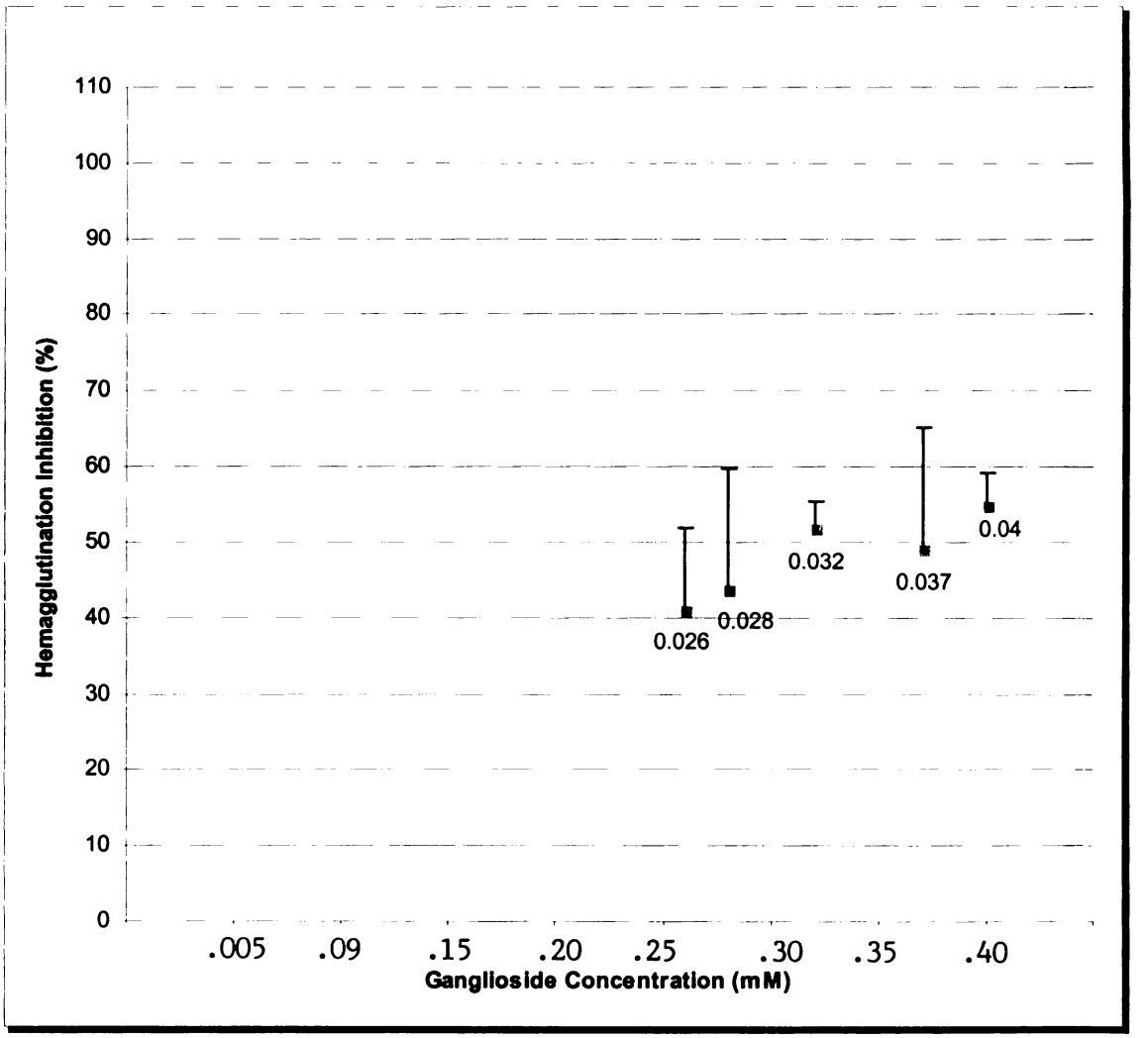


Figure IV.22: Inhibition of chicken erythrocytes with colonization factor antigen-I (CFA-I) by competitive binding with lactic acid bacteria gangliosides. CFA-I (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

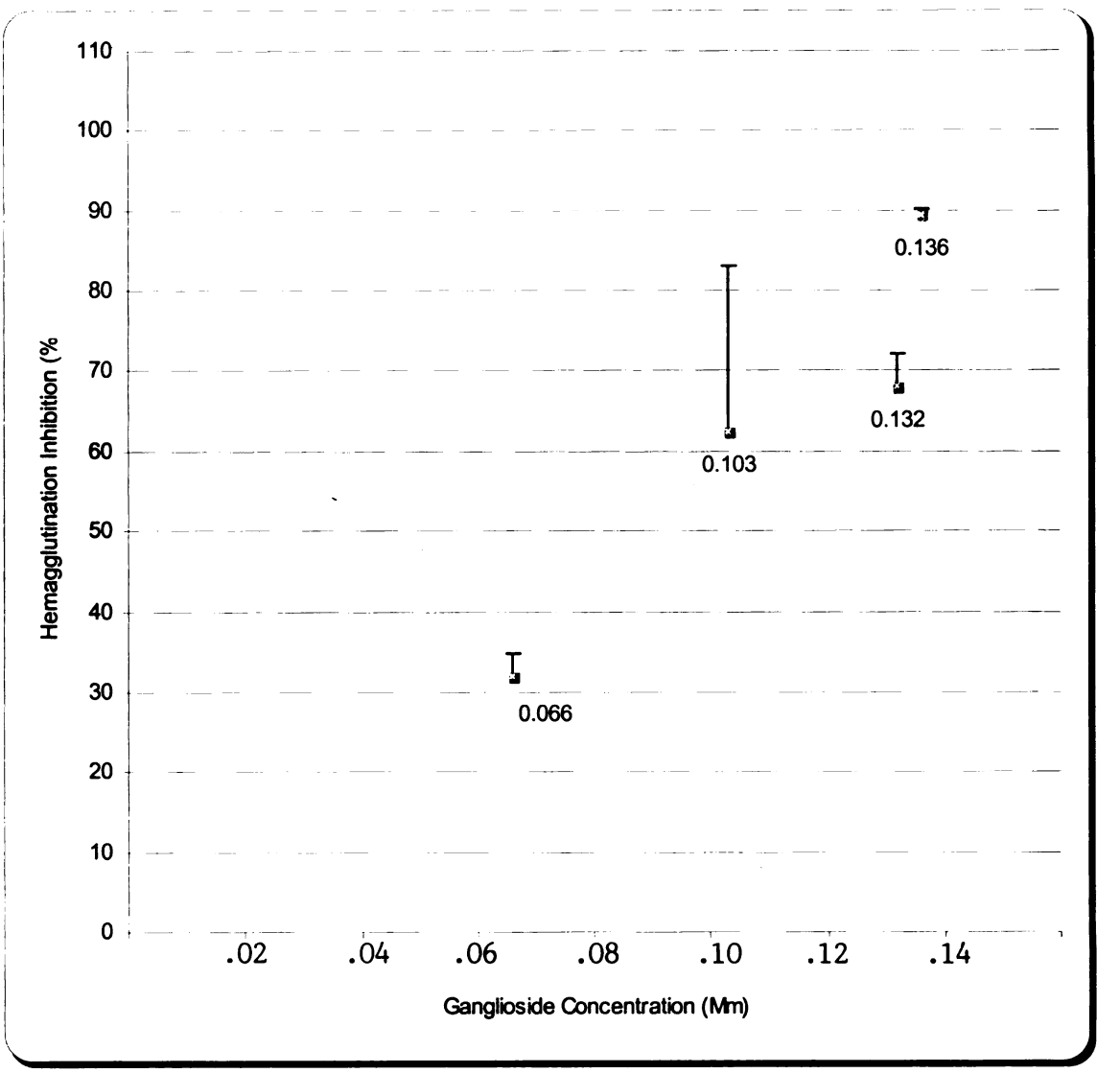


Figure IV.23: Inhibition of bovine erythrocytes with colonization factor antigen-I (CFA-I) by competitive binding with sweet butter milk gangliosides. CFA-I (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

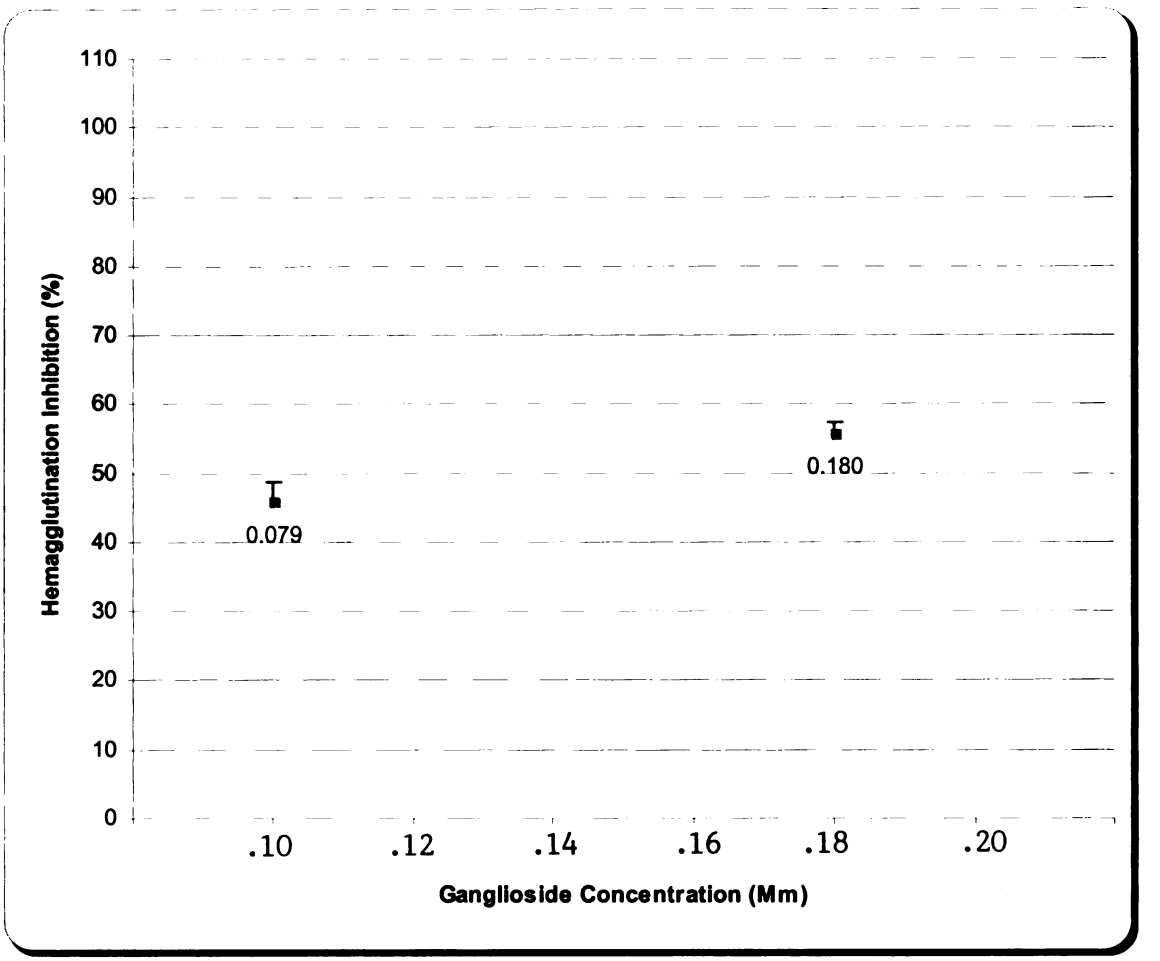


Figure IV.24: Inhibition of bovine erythrocytes with colonization factor antigen-I (CFA-I) by competitive binding with cultured butter milk gangliosides. CFA-I (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

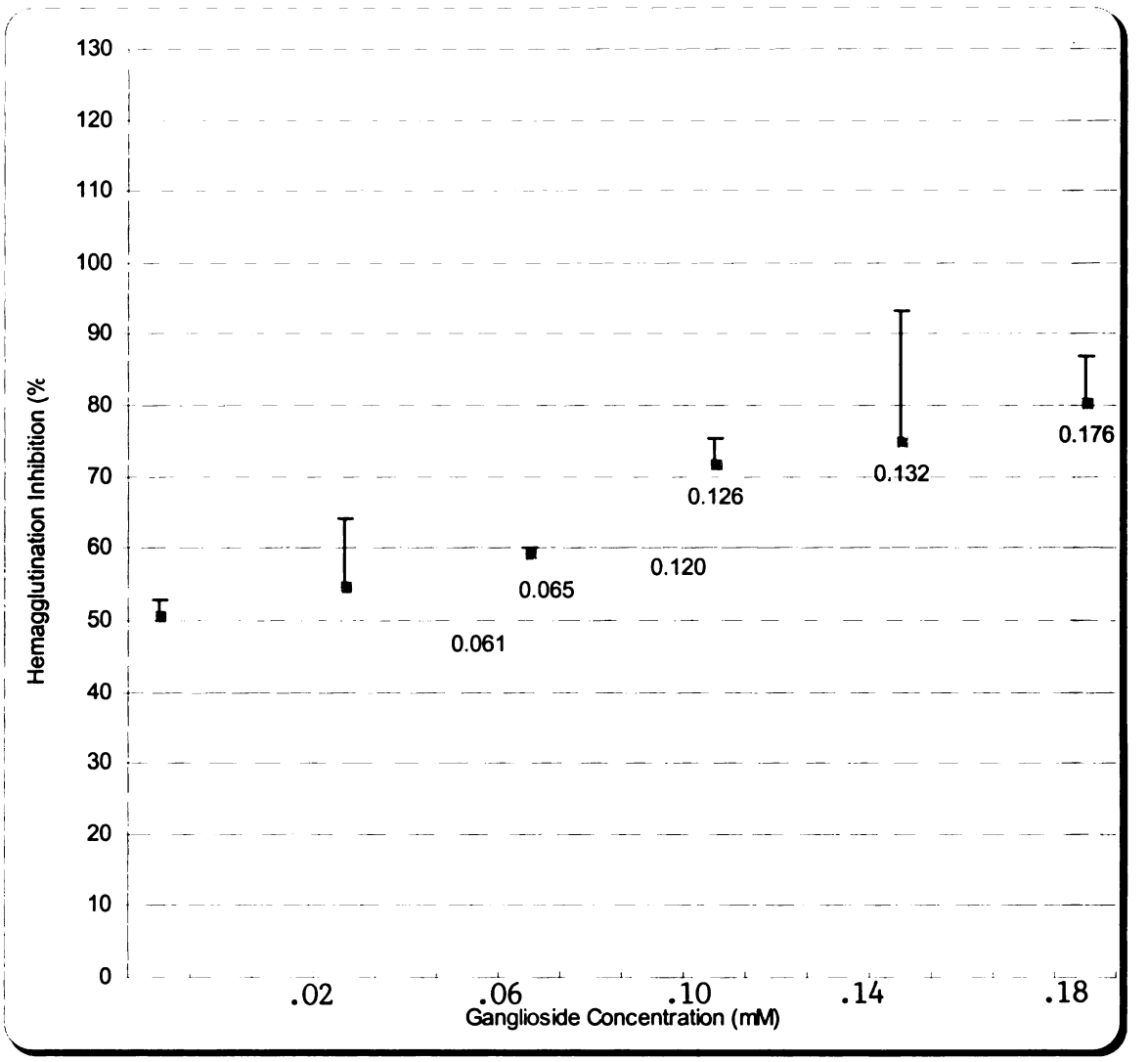


Figure IV.25: Inhibition of bovine erythrocytes with colonization factor antigen-I (CFA-I) by competitive binding with yogurt gangliosides. CFA-I (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

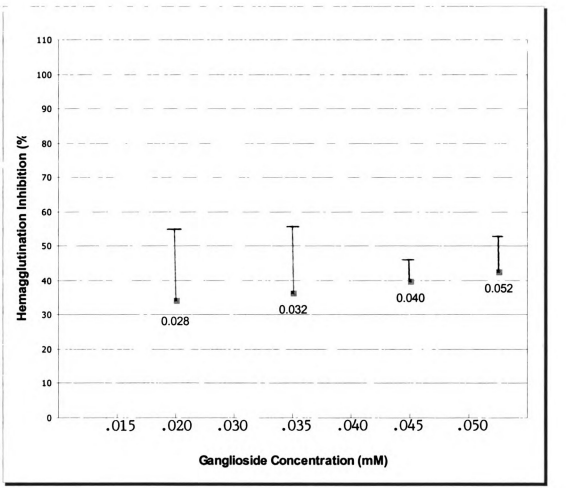


Figure IV.26: Inhibition of bovine erythrocytes with colonization factor antigen-I (CFA-I) by competitive binding with lactic acid bacteria gangliosides. CFA-I (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point.

Additionally, when concentrations of 0.016mM and 0.176mM yogurt gangliosides were compared, the differences between inhibition capabilities were determined to be significant at a confidence level of 0.01 utilizing the t-Test. Lactic acid bacteria mixed ganglioside concentrations (Fig. IV.22) in the range of 0.04 mM to 0.026mM produced inhibitions ranging from 34.3% to 42.7%; Sd ranging from 3.46SdU to 16.07SdU and SE_m ranging from 1.99SE_mU to 9.27Se_mU. When concentrations of 0.026mM and 0.04mM lactic acid bacteria gangliosides were compared, the differences between percent inhibitions were determined to be significant at a confidence level of 0.05 utilizing the t-Test.

Depicted in Figures IV.23 through IV.26 are the results of CFA-I^+ and bovine erythrocytes which produced the following ranges of hemagglutination-inhibition percentages, standard deviations and standard error of the mean. Sweet buttermilk mixed ganglioside concentrations (Fig. IV.23) in the range of 0.066 mM to 0.136mM produced inhibitions ranging from 32% to 89%; Sd ranging from 0.70SdU to 20.46SdU and SE_m ranging from 0.49SE_mU to 1.99Se_mU. Comparing concentrations of 0.066mM and 0.136mM sweet butter milk gangliosides, the differences between percent inhibitions were determined to be significant at a confidence level of 0.01 utilizing the t-Test. Cultured buttermilk mixed ganglioside concentrations (Fig. IV.24) in the range of 0.079mM to 0.180mM produced inhibitions ranging from 46% to 56%; Sd ranging from 1.41SdU to 2.82SdU and SE_m ranging from 0.99SE_mU to 1.99Se_mU. Additionally, when concentrations of 0.079mM and 0.180mM mixed cultured butter milk gangliosides were compared, the differences between percent inhibitions were determined to be significant at a confidence level of 0.01 utilizing the t-Test. Mixed yogurt ganglioside concentrations

(Fig. IV.25) in the range of 0.061 mM to 0.176mM produced inhibitions ranging from 50% to 80.5%; Sd ranging from 1.92SdU to 25.45SdU and SE_m ranging from 1.35SE_mU to 25.99Se_mU . When concentrations of 0.061mM and 0.176mM mixed yogurt gangliosides were compared, the differences between percent inhibitions were determined to be significant at a confidence level of 0.05 utilizing the t-Test. Mixed lactic acid bacteria ganglioside concentrations (Fig. IV.26) in the range of 0.026 mM to 0.052mM produced inhibitions ranging from 34% to 43%; Sd ranging from 4.24SdU to 19.345SdU and SE_m ranging from 2.72SE_mU to 11.31Se_mU. Additionally, when concentrations of 0.026mM and 0.052mM mixed yogurt gangliosides were compared, the percent inhibition of the larger concentration was determined not to be significantly greater at a confidence level of 0.05 utilizing the t-Test.

Hemagglutination assays were initially performed using two different strains of $K99^+$ *E. coli,* one designated $K99^+$-45 *E. coli* and the other $K99^+$-47 *E. coli.* Limited assays using $K99^+$-45 *E. coli* were conducted. When it was determined that the abilities of both strains to inhibit agglutination were comparable, the decision was made to concentrate exclusively on the $K99^+$-47 *E. coli* strain, conserving our limited gangliosides resources for other comparisons. All the results reported are based on assays using $K99^+$-47 E. coli.

Discussion

Enteric diseases, including enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and enterotoxigenic *E. coli* (ETEC), all cause diarrhea, with ETEC being the most common cause of diarrhea in young farm animals (119, 143). Attachment of

enterotoxigenic bacteria to the susceptible host tissue is the initial step in the disease process (119, 159). Two important factors that provide ETEC with means of colonization are the expression of pili, which allow for the attachment of bacteria to the intestinal wall, and the production of enterotoxins that influence intestinal secretions (119). The hemagglutination-inhibition assay has the ability to mimic ETEC pili binding to the intestinal mucosa and therefore is regarded as a good in vitro test system. Currently, the treatment for ETEC centers on rehydration and antibiotic treatment of infected species (12, 14, 27). The extensive use of antibiotics has lead to the development of antibiotic resistant strains of *E. coli* (27, 45). Interest in providing alternatives to the standard treatments for ETEC is an important basis for this research. Carbohydrate moieties on the cell surface of sheep, horse, cow, chicken, and human erythrocytes resemble various gangliosides. These chemical structures have some characteristics similar to the receptors used by pathogenic agents to adhere to the intestinal epithelium. The assay used is based on research previously done by Mouricout et al. (116).

The results generated by this research demonstrate that purified mixed gangliosides from sweet and cultured buttermilk, yogurt, and LAB will inhibit the binding of the livestock pathogen K99$^+$ *E. coli* and the human pathogen *E. coli* CFA-I$^+$ to varying degrees. The mechanism by which binding of the bacterial cell to the red blood cell occurs has been reported to be similar, if not identical, to the binding of the bacterial cell to the intestinal mucosa (116, 124, 158). Therefore, it is reasonable to infer that the adhesion of the enterotoxigenic *E. coli* to the intestinal mucosa, which is a prerequisite for colonization, could be inhibited by the consumption of dairy products that contain gangliosides.

Fujiwara et al. (44) demonstrated that enterobacteria have a high affinity for a specific neutral glycolipid and that this binding can be inhibited through the use of ganglioside-containing bifidiobacteria strains. Other researchers have indicated that gangliosides extracted from LAB also have this bacterial-interference effect (139). The mixed gangliosides contained in cultured dairy foods are contributed by both the bacteria and the milk fat.

Determining why a 10-fold increase in ganglioside amount is needed when using BBG to inhibit agglutination, as compared to mixed gangliosides from LAB and dairy products, has several possible explanations. It is conceivable that the preparation of mixed gangliosides contains proteins that may interfere with bacterial binding. It is also reasonable that, among the mixed gangliosides, one or several possess a higher affinity for the tested bacterial strains as compared to the affinity of BBG. When comparisons have been made between the inhibition of hemagglutination by gangliosides, the specific composition of the ganglioside is extremely important. Enterotoxins of *Vibrio cholerae* will bind to monosialoganglioside-1 with a higher affinity than other tested gangliosides (63). Monosialoganglioside-3 has been reported to be the $K99^+$ receptor by Kyogashima et al (84). The findings of this research demonstrated that mixed ganglioside preparations from sweet and cultured buttermilk, lactic acid bacteria and plain cultured yogurt were more effective as inhibitors of $K99^+$ *E. coli* hemagglutination than GM_1. However, it is important to note that when monosialoganglioside-3 (GM_3) (Fig. IV.8) and bovine brain gangliosides (Fig. IV.7) were compared at a concentration of 0.001mM utilizing sheep erythrocytes, the data indicate that GM_3 does exhibit the ability to inhibit agglutination

more severely than BBG. These findings were consistent using either horse or sheep erythrocytes.

There appears to be no perceptible differences between the inhibiting capacities of sheep erythrocytes and horse erythrocytes with $K99^+$-47 *E. coli*. Sweet buttermilk, cultured buttermilk or yogurt also did not exhibit any major differences as far as their ability to inhibit hemagglutination. It is important to note that the data suggest differences between the inhibitory abilities of commercial gangliosides, (GM_3 and bovine brain gangliosides) and those of gangliosides extracted from dairy products. The ability of mixed gangliosides isolated from dairy products to inhibit agglutination at lower concentrations, when compared to commercially prepared gangliosides, was demonstrated with both horse and sheep erythrocytes.

As discussed earlier, the possibility of the mixed gangliosides from cultured dairy food origins containing gangliosides with a greater affinity for the $K99^+$ pili is a reasonable explanation for this phenomenon. Hemagglutination-inhibition assays using CFA-I^+ were more consistent at specific concentrations and range variations extremely small as compared to tests with $K99^+$ *E. coli*. CFA-I^+ hemagglutination assays also produced similar inhibitions at constant concentrations when comparing cow percentage inhibitions to chicken percentage inhibitions. BBG gangliosides were required in amounts more than 10 times that of extracted mixed gangliosides, which proved to be the case in agglutination tests with $K99^+$ *E. coli*. CFA-I^+ assays utilized only purified gangliosides, which may account for the increase in repeatability.

E. coli $K88^+$, the negative control, produced very low or no hemagglutination inhibition (see Fig. IV.15 through IV.18). The negative percentage inhibitions would

indicate a loss of erythrocytes. The data do not suggest that this was the case. In actuality, the sampling of nonagglutinated erythrocytes was not an exact measurement, but rather an estimate. Precautions were taken: Each assay was run in duplicate, each chamber was sampled twice with an average used as the erythrocyte count, and only one person performed the Coulter Counter sampling. The initial erythrocyte counts may have been somewhat low and, when used in the equation, produced negative numbers for the percentage inhibition of $K88^+$ *E. coli.* It is reasonable to conclude that $K88^+$ did not impede the agglutination of erythrocytes from horse and sheep, and the addition of gangliosides caused no disruption in this process. More tests were performed with $K88^+$ *E. coli* hemagglutination; the percentage inhibitions included in Fig. IV.15 through IV.18 represent an average of three tests at each concentration and also coincide with $K99^+$ *E. coli* concentrations, allowing for more accurate comparisons of the data.

These results underscore and support previous reseach (81, 100) as to the possible benefits of regular consumption of cultured dairy foods in preventing enteric diseases. It was calculated that a person would need to consume three to four 8-ounce containers of yogurt per day to obtain concentrations similar to those shown to be effective in this study.

CHAPTER V

INHIBITION OF ADHESION OF *HELICOBACTER PYLORI* BY MIXED GANGLIOSIDES IN LACTIC ACID BACTERIA, BOVINE MILK FAT, AND CULTURED DAIRY FOODS

Abstract

Gangliosides are a diverse group of naturally occurring, complex sugar-containing glycosphingolipids, which are found in the milk fat of all mammalian species as part of the milk fat globular membrane (2, 14, 51, 79). Gangliosides are also localized on the outer plasma membrane, and studies have shown that cell-surface gangliosides serve as recognition sites for the attachment of viruses, bacteria, and bacterial toxins (126). Recently, the involvement of *Helicobacter pylori (H. pylori)* in gastric diseases has been researched (9, 10, 53, 101, 102). Studies have indicated that *H. pylori* binds to gangliosides, and that the adhesion of the bacteria to glycosphingolipid receptors is an initial step in the pathogenesis and propagation of disease. Mixed gangliosides recovered and purified from cultured dairy products and lactic acid bacteria (LAB) were found to be effective in inhibiting the pilus-mediated adhesion of *H. pylori* to chicken erythrocytes. Ganglioside inhibition was assessed using a mannose-resistant hemagglutination assay. *H. pylori* was preincubated with gangliosides before their exposure to erythrocytes, and nonagglutinated erythrocytes were counted electronically with a Coulter Counter. Ganglioside concentrations in the cultured dairy products and LAB ranging from 0 .126

mM to 30.0 mM resulted in a decrease in hemagglutination ranging between 0% and 79% when *H. pylori* was agglutinated with chicken erythrocytes. On the basis of these results, mixed gangliosides may possibily limit the adhesion of *H. pylori* to the gastric epithelial cells, thereby decreasing the organism's contribution to gastric disease.

Introduction

Peptic-ulcer disease is a chronic inflammatory condition affecting the stomach and duodenum; it is believed to cause complications to at least 10% of the U.S. population at some time during their lives (9, 10, 101, 175). The pathogenesis of this disorder was first believed to be related to stress and diet. Digestive secretions, such as gastric acids, were later targeted as the cause. The role of the bacterium identified as *Helicobacter pylori (H. pylori)* in gastric diseases recently has been under investigation (9, 10, 101).

This bacterium is found almost exclusively in the stomach and duodenum, where it is closely associated with the gastric epithelial cells and/or the mucous layer (9). Evidence that *H. pylori* is involved in the etiology of duodenal and gastric inflammation and ulcers, and that it is also a pathogen causing human gastritis, is compelling (9, 10, 101, 175).

The specific adherence of *H. pylori* to gastric epithelial cells may be critical to maintenance of infection and subsequent pathogenicity. When sialic acid receptors were removed from erythrocytes, the inhibition of the hemagglutination factor of *H. pylori* was achieved (95). This same research paper concluded that the receptor specificity of the *H. pylori* hemagglutin is in the same class as the $K99^{+}$ fimbrial adhesion of enterotoxigenic

E. coli (95). Other studies have indicated that *H. pylori* binds to gangliosides and that the adhesion of the bacteria to glycosphingolipid receptors is an initial step in the pathogenesis and propagation of disease (135, 142, 143). These findings suggested that *H. pylori* might be a good candidate to test for inhibition with extracted mixed gangliosides from cultured dairy foods and lactic acid bacteria (LAB), as assessed by the hemagglutination assay.

Materials and Methods

The purified mixed gangliosides recovered from cultured buttermilk, yogurt, lactic acid bacteria (LAB) and evaporated milk (Carnation) were tested for their ability to inhibit the pilus-mediated adhesion of *H. pylori* to chicken erythrocytes. Red blood cells mixed with bovine brain gangliosides (Sigma Chemical, St. Louis, MO) (BBG), were used as a positive control.

Source of Gangliosides

Mixed ganglioside extractions and purifications were performed in the laboratory of Dr. Kathleen Moore, Department of Chemistry, Oakland University. The purifications were conducted on lactic acid bacteria (LAB) obtained from a buttermilk starter culture (Darigold, Seattle, WA), cultured powdered buttermilk (Darigold, Seattle, WA), and plain cultured yogurt (MSU Dairy Plant, East Lansing, MI), by a modification of the method of Bonafede et al. (14). Specifically, 100 g of dried dairy product (powdered sweet buttermilk, powdered cultured buttermilk, or lyophilized yogurt) was suspended in 1,000 ml of chloroform:methanol (1:1) and allowed to stir overnight. After removal of solid material by Buchner filtration, the extract was subjected to rotary evaporation to remove

solvents. The dried extract was resuspended in 50 ml of water and dialyzed against deionized water for 2 days in a cold box. The dialyzed sample was lyophilized and resuspended in chloroform:methanol:water (C:M:W; 30:60:8) for anion-exchange chromatography. The sample was applied to a 1.5-cm x 30- cm column of DEAE Sephadex A-25 (Sigma Chemical) that was preequilibrated with the C:M:W solvent. The column was then washed with 175 ml of the C:M:W solvent and 75 ml of methanol. Gangliosides were eluded from the column with 150 ml of 0.4 M ammonium acetate in methanol. Ganglioside-containing fractions were pooled, and methanol was removed by rotary evaporation. The dried ganglioside fraction was resuspended in deionized water and dialyzed against deionized water for 2 days to remove ammonium acetate. The dialyzed ganglioside fraction was lyophilized and resuspended in minimal deionized water for quantitation, TLC analysis, and hemagglutination studies.

Starter cultures used for the production of yogurt were obtained from Chris Hansen's laboratory (Milwaukee, WI). The starter culture (R-39) contained multiple strains of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. To produce large quantities of LAB for the isolation of bacterial cell wall gangliosides, 0.2 ml of starter culture was inoculated per liter of MRS Broth (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD) and incubated at 39.5°C for 72 h. The resultant culture yielded approximately 7 g of packed wet cells per liter of medium when harvested by centrifugation at 7,650 for 20 min. Approximately 1,400 g of wet bacterial cells were initially propagated and harvested for ganglioside isolation using this method. The starter culture (8803) for buttermilk, which was obtained from Darigold, Seattle, WA, was also

grown up. The bacterial strains used in this starter culture were proprietary; therefore, specific bacterial identification was not possible.

Ganglioside Quantitation

Thin layer chromatography (TLC) was performed using silica gel 60 TLC precoated plates (glass plates 10 x 10cm Merck). The plates were activated at 100° C and ganglioside preparations and ganglioside and N-acetylneuraminic acid (NANA) standards were spotted on silica gel G plates and placed in a chamber containing chloroform: methanol:0.25% $CaCL_2$ solution (30:18:4). Plates were developed in the chambers for twice the time it took the solvent to reach the top of the plate. Plates were then removed, dried, sprayed with resorcinol-HCL for visualization, and heated at 110°C for 15 min. Sialic-acid-containing material gave blue spots; organic material lacking sialic acid charred brown. Densitometry of TLC plates was performed with a Shimadzu Dual-Wavelength TLC-Scanner CS930. The chromatograms were obtained by single wavelength zig-zag scanning of each lane at 620 nm (beam dimension: 0.4 X 0.4mm) in the reflection mode (168). The purified preparations yielded blue bands of varying patterns. All types of preparations had a band that migrated with standard GD_3; LAB preparations had only one additional band, whereas the dairy products had six to eight separate ganglioside bands. The yields resulting from this process were small, but the preparations were highly purified and devoid of extraneous contamination.

Source of Erythrocytes

Erythrocytes were obtained from chickens located at the Michigan State University, (East Lansing, MI) poultry farm facility. Twenty five milliliters of whole blood was drawn from chickens weekly. Freshly drawn blood was immediately aliquoted

to prelabeled chilled test tubes containing an equal volume of Alsever's Solution (Sigma Chemical, St. Louis, MO). Test tubes were inverted four times for mixing and placed in an ice bath during transportation. Erythrocytes used for procedures were never older than one week and remained stored at 4°C .

Source of Pathogens and Growth Conditions

Helicobacter pylori (strain ATCC 43504, NCTC 11637) cultures were provided by the lab of Dr. Raul Hollingsworth in the Department of Biochemistry, Michigan State University (MSU). *H. pylori* was grown in Minca broth with IsoVitaleX (BBL/Becton Dickinson, Cockeysville, MD). The cultures were incubated aerobically at 37°C in a shaking water bath prior to use.

Bacterial Cell Wall Gangliosides

The bacterial cell wall gangliosides were isolated and purified at Oakland University in the laboratory of Dr. Kathleen Moore. With respect to the isolation of ganglioside from LAB, the typical preparation involves motor-driven homogenization of 60 g of bacterial cells (wet weight) with 100 ml of sucrose phosphate buffer, pH 7.4. The mixture was then transferred into a chilled BeadBeator chamber (Fisher Scientific, Atlanta, GA). The bacteria cells were ruptured by glass beads for three 1-min intervals; each 1-min process was followed by a 10-min cool-down. The process was done in a cold box. After treatment was complete, the beads were allowed to settle before decanting of the homogenate. The beads were washed twice with 50 ml of buffer; the washes were then combined with the homogenate.

The homogenized bacterial mix was centrifuged for 20 min at 25,000 revolutions per minute (RPM). This procedure pelleted the bacterial membranes, which contained

most of the gangliosides. The supernatant was lyophilized and then extracted for its minimal ganglioside content. The bacterial-membrane pellets were weighed and gradually homogenized with chloroform:methanol (1:1 v/v, 20 ml of solvent per gram of pellet). After overnight extraction, the process continued as previously described, according to the method of Bonafede et al. (14). Eventually, lyophilized samples were extracted by Dr. Moore's laboratory using the method described earlier.

Hemagglutination-Inhibition Assay

The crude mixed gangliosides recovered from sweet and cultured buttermilk, yogurt, lactic acid bacteria (LAB) and evaporated milk were tested for their ability to inhibit the pilus-mediated adhesion of *H. pylori* to chicken erythrocytes. Chicken erythrocytes mixed with bovine brain gangliosides (BBG) were used as a positive control. The use of hemagglutination (HA) in the presence or absence of p-mannose and its analogs provides a basic distinction between mannose-sensitive (MS) and mannose-resistant (MR) adhesions and is often used as one form of classification. Enterotoxigenic *E. coli* possesses MR pili, which means they are not inhibited by mannose, and it was therefore included as part of the phosphate buffer.

One hundred microliters of *H. pylori* (10^8/ml) were mixed with 200 μm of mixed gangliosides; both gangliosides and *H. pylori* were previously washed in 0.14 M of phosphate-buffered saline (pH 6.8) containing 0.1 M of alpha-methylmannoside (PBSM), and incubated at 4°C for 30 min. Then 100 μl of chicken erythrocytes were added and the mixture was incubated for an additional 60 min at 4°C. Clumping of erythrocytes or hemagglutination was counted in 20 ml of Isoton (Coultronics, Margency, France) with

an electronic particle counter (Coulter Counter, Coultronics, Margency, France, tube orifice 100 µl). All statistical calculations were performed using the Microsoft Excel 2000 statistical package. The percentage of hemagglutination inhibition was calculated by the following standardized formula (116):

$$\frac{RBC_f \text{ in inhibition test} - RBC_f \text{ in 100\% MRHA}}{RBC_f \text{ 0\% MRHA} - RBC_f \text{ in 100 MRHA}} \times 100$$

where:

RBC = Red blood cells

MRHA = Mannose resistant hemagglutination

RBC_f in inhibition test = 100 µl RBC, 200 µl ganglioside, 100 µl bacteria

RBC_f in 100% MRHA = 100 µl RBC, 200 µl buffer, 100 µl bacteria

RBC_f in 0% MRHA = 100 µl RBC, 300 µl buffer

Results

The hemagglutination-inhibition assay has the ability to mimic *H. pylori* pili binding to the gastric epithelial cells and therefore serves as a good *in vitro* test system. The hemagglutination-inhibition assay is depicted diagrammatically in Figure IV.1 and Figure IV.2 is a photograph of the hemagglutination-inhibition assay. The percentage of hemagglutination inhibition was calculated using a standardized formula (116). Purified mixed gangliosides extracted from LAB and cultured buttermilk, yogurt and evaporated milk will inhibit the pilus-mediated hemagglutination of *H. pylori* to chicken erythrocytes.

Hemagglutination-inhibition results representing the means of percentage inhibitions at specific concentrations utilizing *H. pylori* with chicken erythrocytes are shown in Figures V.1 through V.4. As shown in these figures, mixed gangliosides from cultured buttermilk, yogurt, lactic acid bacteria and evaporated milk were effective in inhibiting *H. pylori* binding to chicken erythrocytes at concentrations ranging from 0.061 mM to 0.79 mM. Hemagglutination was decreased between 1% and 79% using these mixed gangliosides. Each mean inhibition represents a minimum of two replications at this concentration.

As shown in Fig. V.1 the use of chicken erythrocytes with extracted mixed gangliosides from lactic acid bacteria produced the following hemagglutination inhibition percentages, standard deviations and standard errors of the mean. Lactic acid bacteria ganglioside concentration of 0.079mM produced 0% hemagglutination inhibition (HI); a standard deviation (Sd) of 0.0 and a standard error of the mean (Se_m) of 0.0. Lactic acid bacteria ganglioside concentration of 0.103mM produced 46% HI; 11.31 Sd and 7.99 Se_m. Lactic acid bacteria ganglioside concentration of 0.132mM produced 48% HI; 16.97 Sd and 11.99 Se_m. Lactic acid bacteria ganglioside concentration of 0.136mM produced 54% HI; 9.89 Sd and 6.99 Se_m. Additionally, when concentrations of 0.079mM and 0.136mM lactic acid bacteria gangliosides were compared, the percent inhibition of the larger concentration was determined to be significantly greater at a confidence level of 0.01 utilizing the t-Test.

As shown in Fig. V.2 the use of chicken erythrocytes with extracted mixed gangliosides from cultured buttermilk produced the following hemagglutination inhibition percentages, standard deviations and standard errors of the mean. Cultured

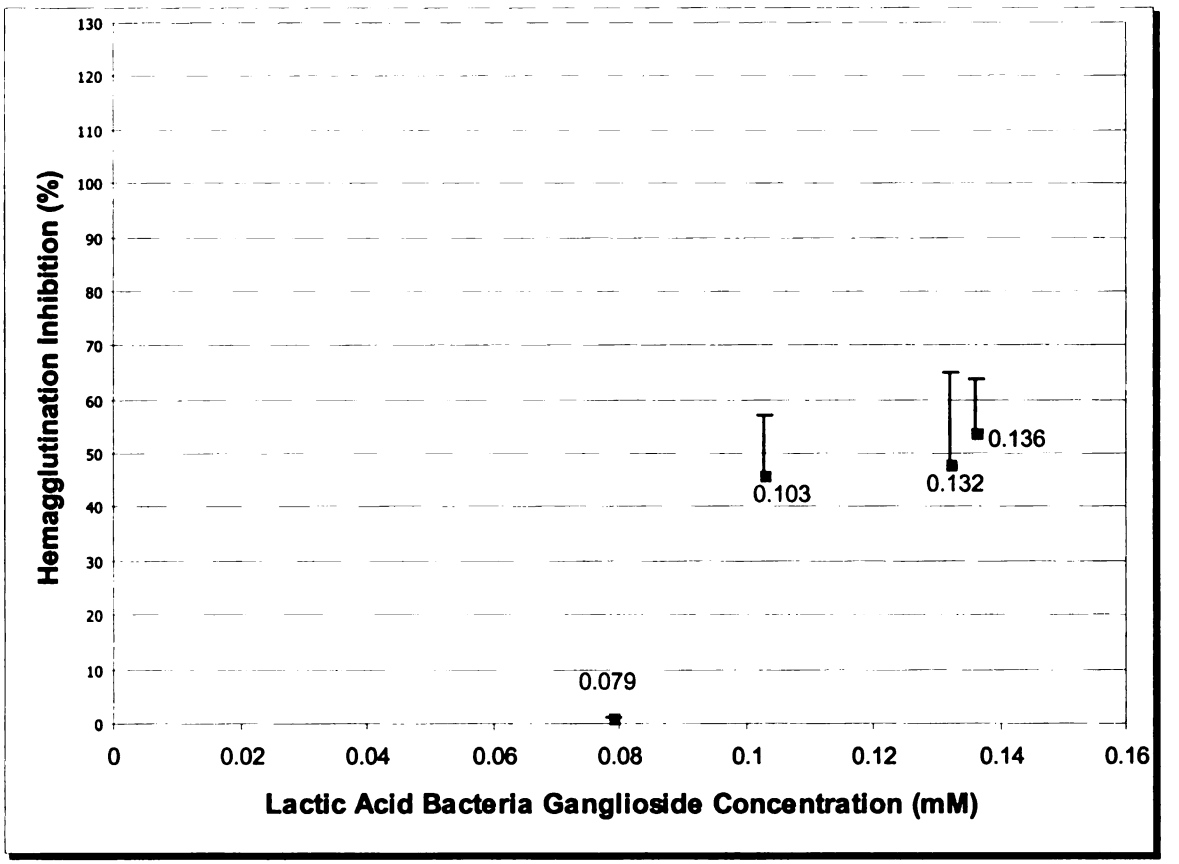


Figure V.1: Inhibition of chicken erythrocytes with *Helicobacter pylori* by competitive binding with lactic acid bacteria gangliosides. *H. pylori* (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point. Ganglioside quantitation required that preparations and standards be spotted on silica gel G plates (Merck), placed in a chamber containing chloroform: methanol:0.25% $CaCL_2$ solution (30:18:4); developed; removed, dried, sprayed with resorcinol-HCL for visualization.

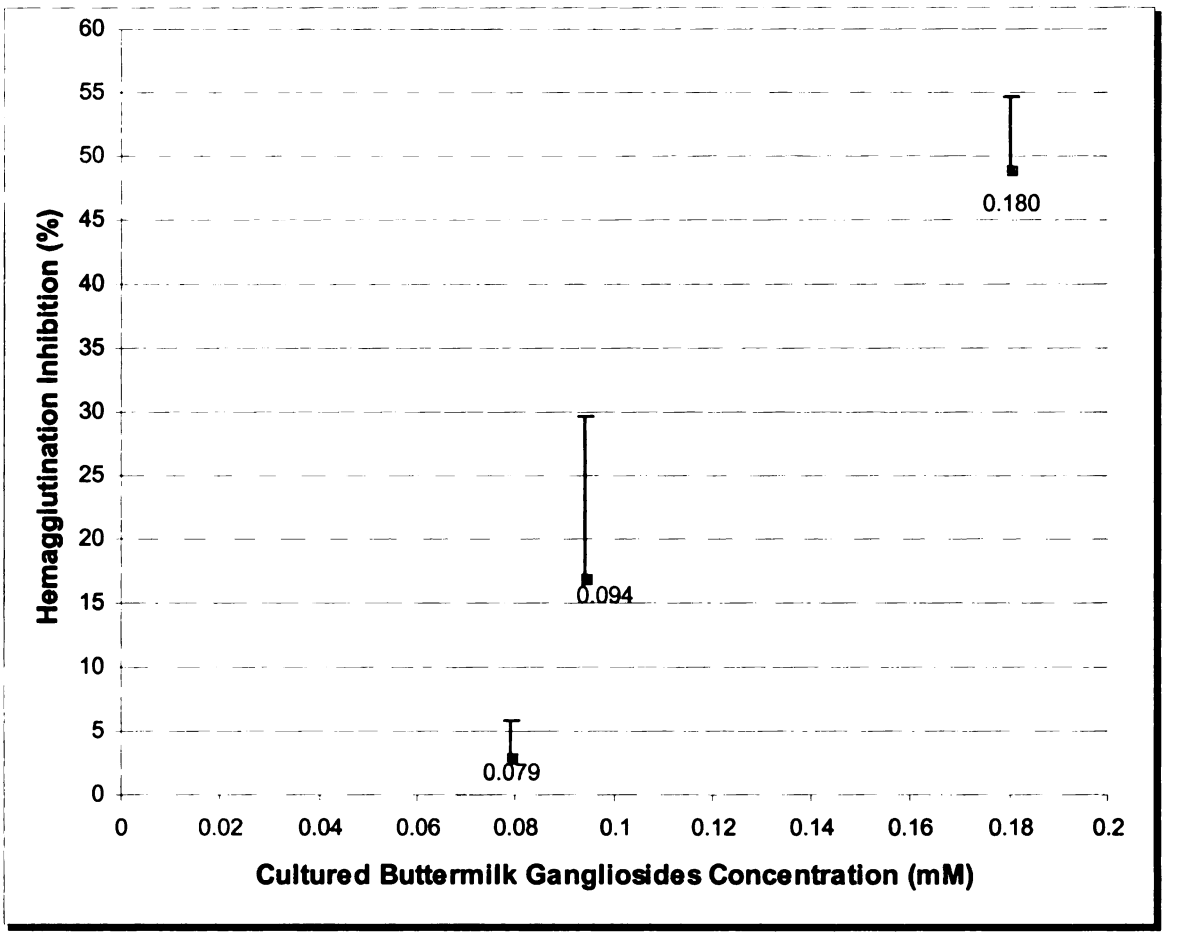


Figure V.2: Inhibition of chicken erythrocytes with *Helicobacter pylori* by competitive binding with cultured buttermilk gangliosides. *H. pylori* (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point. Ganglioside quantitation required that preparations and standards be spotted on silica gel G plates (Merck), placed in a chamber containing chloroform: methanol:0.25% $CaCL_2$ solution (30:18:4); developed; removed, dried, sprayed with resorcinol-HCL for visualization.

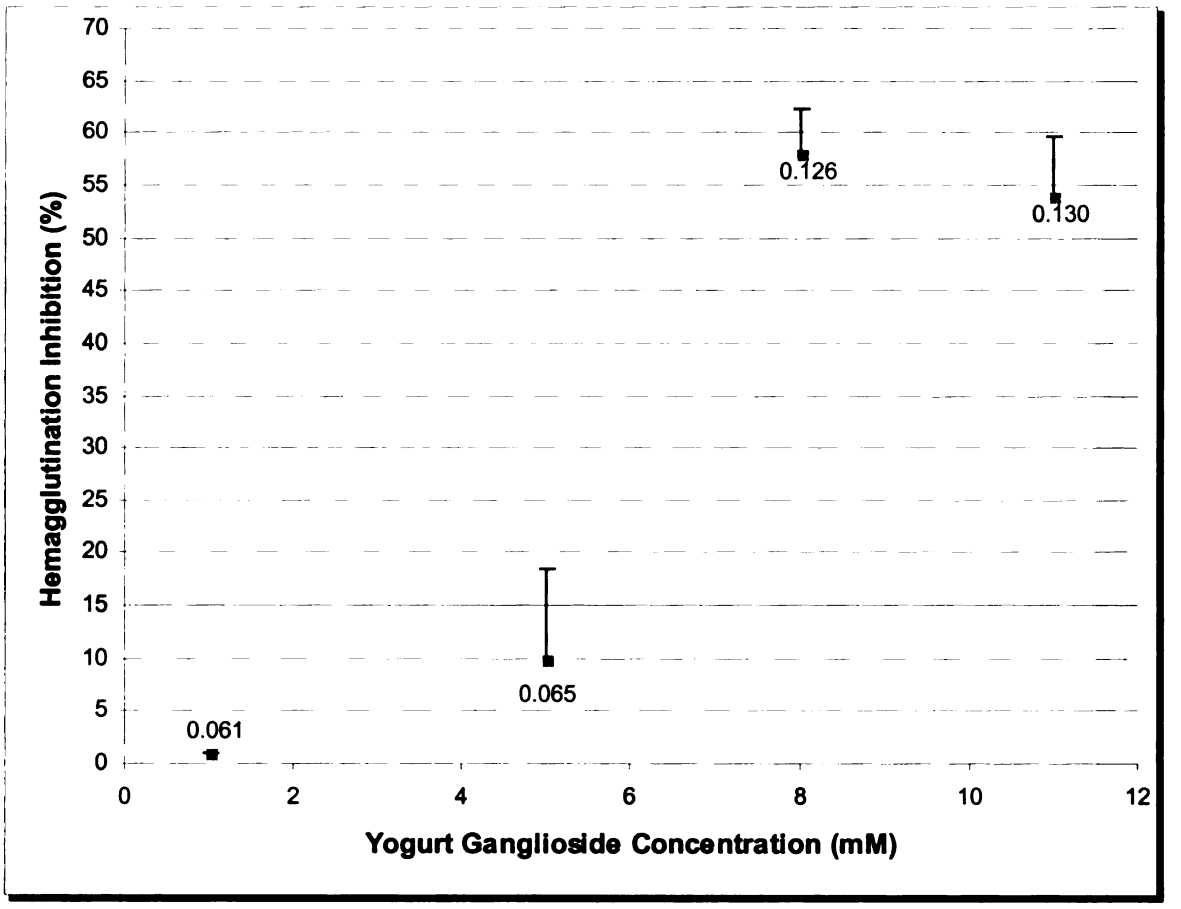


Figure V.3: Inhibition of chicken erythrocytes with *Helicobacter pylori* by competitive binding with yogurt gangliosides. *H. pylori* (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point. Ganglioside quantitation required that preparations and standards be spotted on silica gel G plates (Merck), placed in a chamber containing chloroform: methanol:0.25% $CaCL_2$ solution (30:18:4); developed; removed, dried, sprayed with resorcinol-HCL for visualization.

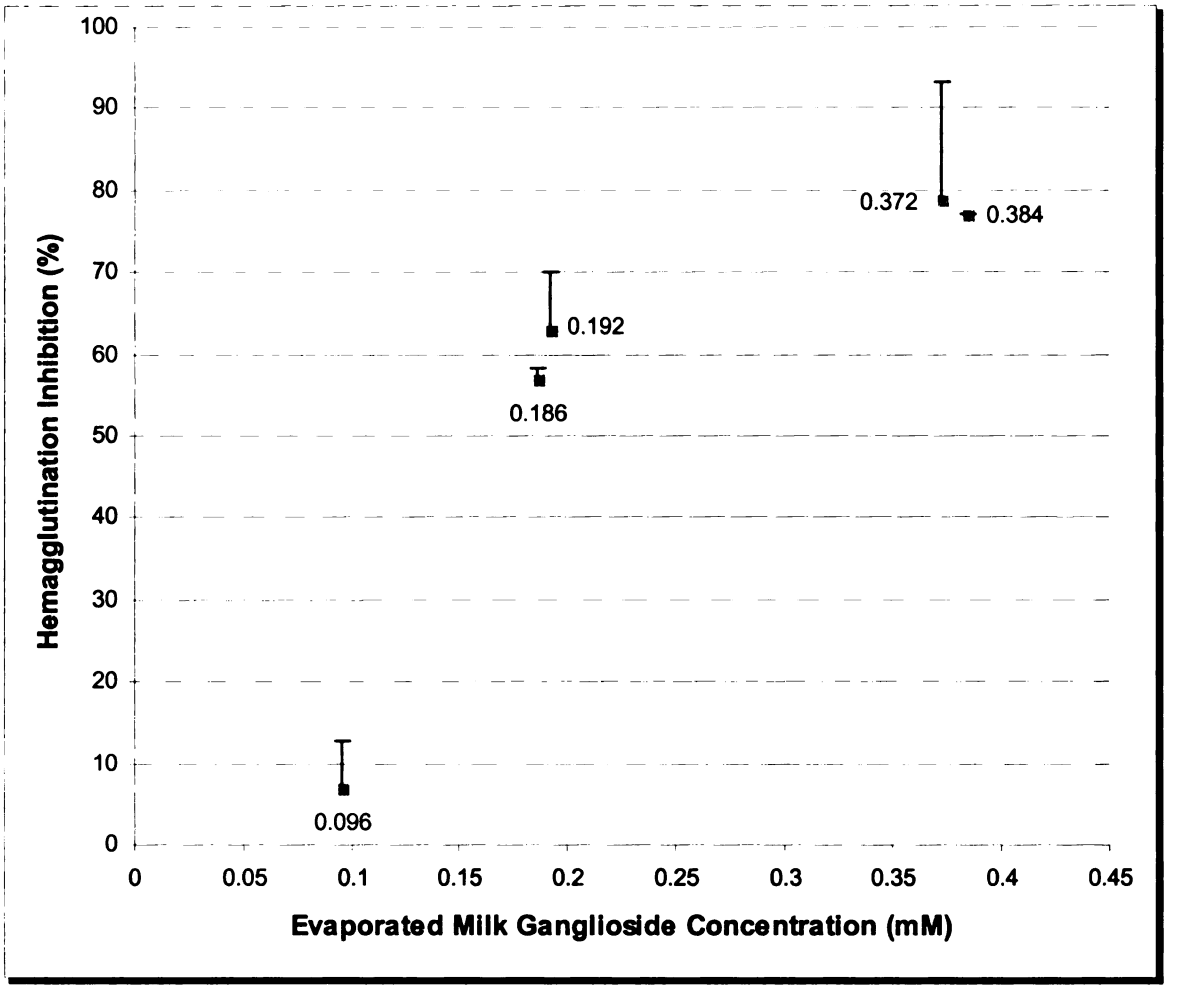


Figure V.4: Inhibition of chicken erythrocytes with *Helicobacter pylori* by competitive binding with mixed gangliosides from evaporated milk. *H. pylori* (10^8/ml) were preincubated with gangliosides for 30 min. Erythrocytes were added and incubated an additional hour. Percent inhibitions were calculated using a standard equation based on Coulter Counter measurements. All values are means with a minimum of two replications at each concentration. Standard deviation is represented by the ascending lines at each point. Ganglioside quantitation required that preparations and standards be spotted on silica gel G plates (Merck), placed in a chamber containing chloroform: methanol:0.25% $CaCL_2$ solution (30:18:4); developed; removed, dried, sprayed with resorcinol-HCL for visualization.

buttermilk ganglioside concentration of 0.079mM produced 3% HI; 2.82 Sd and 1.99 Se_m. Cultured buttermilk ganglioside concentration of 0.094mM produced 17% HI; 12.72 Sd and 8.99 Se_m. Cultured buttermilk ganglioside concentration of 0.180mM produced 49% HI; 5.65 Sd and 3.99 Se_m. Additionally, when concentrations of 0.079mM and 0.18mM cultured buttermilk gangliosides were compared, the percent inhibition of the larger concentration was determined to be significantly greater at a confidence level of 0.01 utilizing the t-Test.

As shown in Fig. V.3 the use of chicken erythrocytes with extracted mixed gangliosides from yogurt produced the following hemagglutination inhibition percentages, standard deviations and standard errors of the mean. The yogurt ganglioside concentration of 0.061mM produced 1% HI; 0.0 Sd and 0.0 Se_m. The yogurt ganglioside concentration of 0.065mM produced 10% HI; 8.48 Sd and 5.99 Se_m.The yogurt ganglioside concentration of 0.126mM produced 58% HI; 4.24 Sd and 2.99 Se_m. The yogurt ganglioside concentration of 0.130mM produced 54% HI; 5.65 Sd and 3.99 Se_m. Additionally, when concentrations of 0.065mM and 0.130mM yogurt gangliosides were compared, the percent inhibition of the larger concentration was determined to be significantly greater at a confidence level of 0.01 utilizing the t-Test.

As shown in Fig. V.4 the use of chicken erythrocytes with extracted mixed gangliosides from evaporated milk produced the following hemagglutination inhibition percentages, standard deviations and standard errors of the mean. The evaporated milk ganglioside concentration of 0.096mM produced 7% HI; 5.65 Sd and 3.99 Se_m. The evaporated milk ganglioside concentration of 0.186mM produced 57% HI; 1.41 Sd and 0.99 Se_m. The evaporated milk ganglioside concentration of 0.192mM produced 63% HI;

7.07 Sd and 4.99 Se_m. The evaporated milk ganglioside concentration of 0.372mM produced 79% HI; 14.14 Sd and 9.99 Se_m. The evaporated milk ganglioside concentration of 0.384mM produced 77% HI; 0.0 Sd and 0.0 Se_m. Additionally, when concentrations of 0.096mM and 0.384mM evaporated milk gangliosides were compared, the percent inhibition of the larger concentration was determined to be significantly greater at a confidence level of 0.01 utilizing the t-Test.

Discussion

The correlation of *H. pylori* infections with peptic ulcer disease has been reported (9, 10, 53, 101, 131, 135, 175). Marshall and others (9, 10, 101, 102, 131) demonstrated that *H. pylori* is responsible for at least 70% of ulcers. Stress, and other causes, such as prescription drug use may play a role, but these are not usually the direct cause of ulcers (9, 10, 131). Additionally, it is important to note that the presence of *H. pylori* does not indicate peptic ulcer formation will occur, but it is unlikely to detect the conditions of peptic ulcer disease or gastritis without this bacterium's presence. Previously, ulcers were treated with a combination of diet alterations and stress reductions. These regimes usually provided only limited relief. With the indication of *H. pylori* as an important causative agent in the etiology of gastric ulcers, antibiotic therapy is currently the course of choice. These antibiotics have proven to be extremely effective in the treatment and cure of gastric ulcers.

Adhesion of bacteria in general and *H. pylori* specifically, to host epithelial surfaces is usually the first step in the colonization process (119, 123, 131, 146, 175). This binding can also cause to induce the expression of virulence factors (146). The

binding of *H. pylori* to erythrocytes has been reported to be via sialoprotein containing the N-acetylneuraminyl-lactose (2-3) linkage in the same class as the K99 fimbrial adhesion of enterotoxigenic *Escherichia coli* (ETEC) (3, 95, 108, 157). *H. pylori* isolates have also been shown to bind strongly with rabbit, guinea pig, chicken, horse, cow, sheep, and monkey erythrocytes (95, 157). These research findings were the basis for the decision to test whether *H. pylori* would bind with the mixed gangliosides from cultured dairy foods in a hemagglutination-inhibition assay.

The specific course of *H. pylori*-induced illnesses are complex. Motility appears to be directly related to the ability to penetrate the epithelial mucus layer. Attachment of the bacterium appears to prevent or severely impair mucus production, allowing this area to be exposed to digestive gastric enzymes. The indication that adherence of *H. pylori* to gastric epithelial cells may be critical to maintenance of infection and ultimate pathogenicity also lends support to the approach of limiting *H. pylori* adherence. It is relevant to indicate that *H. pylori* was demonstrated to have a high binding affinity for the form of N-acetylneuraminyl-lactose which predominates in bovine colostrum (108). The ability of extracted mixed gangliosides from dairy foods to inhibit the adhesion of chicken erythrocytes to *H. pylori* pili indicates that gangliosides extracted from cultured buttermilk, yogurt, LAB, and evaporated milk may provide a useful therapy for combating gastric diseases through the reduction of *H. pylori* gastric epithelial cell adherence.

The substantial evidence which suggests that LAB have an inhibitory effect on pathogenic bacteria in the intestinal tract (18, 32, 35, 45, 49, 50, 51, 59, 62, 63, 90, 103, 109, 117, 185, 189) is generally attributed to the theory of competitive exclusion. This

theory maintains that LAB control the growth and colonization of opportunistic enteric pathogens by effectively competing for nutrients, lowering the luminal pH through the production of lactic acid and the possible production of antibiotic-like substances. *H. pylori* possesses characteristic enzyme activity with urease being of special interest. Urease activity facilitates the production of ammonia from gastric juice urea. The ammonia functions as a base and raises the pH of the gastric environment, which allows for the survival of *H. pylori* in the acidic environment of the stomach. As described earlier, LAB control the growth and colonization of opportunistic enteric pathogens by lowering the luminal pH through the production of lactic acid. In addition to preventing/reducing epithelial binding, LAB can also adversely affect the gastric pH, providing a less stable environment for *H. pylori.*

Although these studies were limited in scope–only two agglutination tests at each concentration were performed–the goal of these experiments was to assess whether cultured dairy foods and LAB could inhibit the hemagglutination of RBC by *H. pylori.* In this respect, the studies were successful. Statistical comparisons were made but their relevance may be over or under stated due to the of the lack of repetitive experiments. There appeared to be no difference between the inhibition capabilities of lactic acid bacteria, yogurt or evaporated milk with respect to similar concentrations. There were significant differences when the smallest concentrations of specific gangliosides were compared to the highest concentrations of these same gangliosides. The results therefore indicate, that as mixed ganglioside concentrations increase, the resultant percentage inhibition also increases.

Present knowledge about the chemical nature of the receptors indicates that they are composed of glycolipids and/or glycoproteins. Various glycoproteins and glycolipids may be able to inhibit the binding of piliated *E. coli* to brush borders, epithelial cells, or erythrocytes (116, 120, 183). The N-acetylneuraminic acid portion appears to play an important role in the inhibition of binding. The hemagglutination-inhibition assay mimics ETEC pili binding to the intestinal mucosa and therefore serves as a good test system.

The results of this research demonstrate that mixed gangliosides from cultured buttermilk, yogurt, LAB, and evaporated milk will inhibit the binding of the human pathogen *H. pylori* to chicken erythrocytes. Therefore, it is reasonable to infer that the adhesion of *H. pylori* to gastric epithelial cells could be inhibited by the consumption of dairy products that contain gangliosides. Additionally, it has been reported that *H. pylori* is rarely seen in children. Although, the literature does not indicate an explanation for this phenomenon, it is important to note that children are proliferate consumers of dairy products. These results underscore the benefits of regular consumption of cultured dairy foods in preventing enteric diseases. It was calculated that a person would need to consume three to four 8-ounce containers of yogurt per day to obtain concentrations similar to those shown to be effective in this study.

CHAPTER VI

SUMMARY

The basic premise of this research is that mixed gangliosides extracted from sweet buttermilk, cultured buttermilk, yogurt and lactic acid bacteria possess antimicrobial activity and enterotoxin inhibiting capabilities that will inhibit the pilus-mediated adhesion of bacteria and that these gangliosides also possess bacteriostatic and bacteriocidal capabilities. There is substantial evidence that lactic acid bacteria (LAB) have an inhibitory effect on pathogenic bacteria in the intestinal tract (49, 50, 62, 103, 109, 178). Several theories have been proposed for this bacterial antagonism; the most widely accepted theory is that of competitive exclusion. This theory holds that LAB control the growth and colonization of opportunistic enteric pathogens by effectively competing for nutrients, lowering the luminal pH by producing lactic acid, and possibly producing antibiotic-like substances. When an imbalance occurs in this arrangement, pathogens gain an advantage, ultimately resulting in a disease state. The antimicrobial activity beneficially modifies the microflora and microbial activity of the intestinal tract serving as a prophylaxis against enteric diseases.

Gangliosides are a diverse group of naturally occurring, sugar-containing glycosphingolipids having sialic acid (N-acetylneuramic acid) as an essential component and a hydrophobic lipid component (ceramide) (88). Studies have shown that cell-surface

gangliosides serve as recognition sites for the attachment of viruses, bacteria, and bacterial toxins (71, 75, 76, 87, 88, 99). This attachment is attributed to the sialic acids and oligosialyl structures in glycoproteins, glycolipids, and the complex polysaccharides associated with the cell walls of microorganisms.

Gangliosides of different types naturally occur in the milk fat of all mammalian species as part of the milk fat globular membrane, which is derived from the plasma membrane of epithelial cells in the mammary gland (2, 14, 51, 79, 170). The gangliosides in milk will inhibit the enterotoxins of *V. cholera* (63) and *E. coli* (12), with human milk gangliosides showing a considerably higher (5-10x) inhibitory effect than bovine or formula milk (45, 51, 57). The difference in activity has been attributed to the ganglioside composition in human and bovine milk (85). It is also important to note that changes in ganglioside composition occur in human and bovine milk throughout the lactation cycle, which may affect the inhibitory ability of these gangliosides (170).

Throughout the world, ETEC is an important cause of neonatal diarrhea in pigs, calves, and lambs, as well as in humans (64, 119, 143). *E. coli* and salmonella are the most common causes of diarrhea in children in developing nations (15, 28, 119). These illnesses are still a major cause of infant and childhood morbidity and mortality. Two virulence determinants are necessary for ETEC to cause diarrhea: enterotoxin production and pilus production. Pili allow *E. coli* to attach to specific receptors on the mucosal surface of the small intestine (31, 32, 38, 42, 158).

The working hypothesis of this project was that mixed gangliosides extracted from dairy products will inhibit the pilus-mediated adhesion of bacteria and that these

gangliosides also possess bacteriostatic and bacteriocidal capabilities. The specific research objectives are as follows:

1. To extract gangliosides from sweet buttermilk, cultured buttermilk, yogurt and lactic acid bacteria.
2. To test extracted mixed ganglioside fractions from sweet buttermilk, cultured buttermilk, yogurt and lactic acid bacteria for enterotoxin inhibiting capabilities.
3. To screen extracted mixed ganglioside fractions from sweet buttermilk, cultured buttermilk, yogurt and lactic acid bacteria for antimicrobial activity using human and animal enterotoxigenic *E. Coli* strains.

One of the most important impediments to ganglioside research is the inability to extract large amounts of crude mixed gangliosides for analysis as well as the ultimate purity level (lack of contaminants) of these gangliosides. In order to achieve the first objective, the development of precise procedures, based on ganglioside extraction from tissue (168) and bovine buttermilk (14), and adapted to sweet and cultured buttermilk, yogurt, evaporated milk and LAB from starter cultures, had to be accomplished. Mixed gangliosides were extracted from cultured dairy foods (cultured buttermilk, yogurt), sweet buttermilk, LAB, and evaporated milk using a modification of the method of Bonafede et al. (14). The extraction process, which was labor intensive and had not previously been developed specifically for cultured dairy foods (CDF), bovine milk fat (BMF), or LAB, required the application of existing protocols to the unique and specialized challenges of ganglioside extractions from these products. In addition to the extraction process, mixed gangliosides were purified with a DEAE- Sephadex A-25 (Sigma Chemical) column,

producing samples with fewer contaminants. Although LAB ganglioside extraction initially was undertaken at MSU, the major task of ganglioside extraction from dairy products (cultured buttermilk, sweet buttermilk, yogurt, evaporated milk) and LAB was undertaken at Dr. Kathleen Moore's laboratory in the Department of Chemistry at Oakland University. Thin layer chromatography (TLC) was performed using silica gel 60 TLC precoated plates (glass plates 10 x 10cm Merck), in order to quantify gangliosides. The plates were activated; ganglioside preparations and ganglioside and N-acetylneuraminic acid standards were spotted on silica gel G plates which were then developed. Plates were removed, dried, sprayed with resorcinol-HCL for visualization, and heated at 110°C for 15 min. Sialic-acid-containing material gave blue spots; organic material lacking sialic acid charred brown. Densitometry of TLC plates was performed with a Shimadzu Dual-Wavelength TLC-Scanner CS930. The chromatograms were obtained by single wavelength zig-zag scanning of each lane at 620 nm (beam dimension: 0.4 X 0.4mm) in the reflection mode (168). The purified preparations yielded blue bands of varying patterns. The qualitative evaluation of several protocols by examining TLC plates provided information as to ganglioside composition of the samples (which were predominantly GD_3), and the ability of these protocols to provide gangliosides with the least contamination. The yields resulting from this process were small, but the preparations were highly purified and devoid of extraneous contamination. The success of these extractions has proven and will continue to be an important part of future analysis in the area of milk-product ganglioside research. The use of purified preparations also provided more consistent results with the hemagglutination assays and the double agar overlay technique.

The extraction of LAB gangliosides indicated that substantially more gangliosides are present in these bacteria than in dairy products (sweet or cultured buttermilk, or yogurt). Of the dairy products tested, yogurt had the highest ganglioside content. The calculation of ganglioside content on a nmol/g dry-weight basis, using purified preparations, produced the following results: cultured buttermilk 7.3 ± 5.0 nmol/g, sweet buttermilk 11.6 ± 8.1 nmol/g, yogurt 13.2 ± 5.6 nmol/g, yogurt starter culture 26.7 ± 8.5 nmol/g, and buttermilk starter culture 65.0 ± 13.7 nmol/g. The use of cultured plain yogurt whose commercial inoculant was also used to grow LAB provided a unique situation in attempting to determine the proportion of the contribution LAB may make to the final ganglioside concentration. This was the premise for using the starter culture. No definitive answers were obtained as to the relationship between the presence of gangliosides in the starter culture to the amount of ganglioside present in the final product, (cultured dairy foods).

Testing gangliosides isolated from actual milk products will likely provide information not previously available, and is important in respect to both objectives 2 and 3. The discovery and subsequent isolation and characterization of ganglioside 9-0-acetyl-GD_3 (14) from bovine buttermilk is an important indication of what further research on actual products could produce. The isolation of this unique ganglioside suggests that LAB may be involved in the modification of milk fat gangliosides or the production of other gangliosides not present in the original milk fat.

Rueda et al. (139) determined the effect on preterm infants of supplementing an adapted milk formula with gangliosides at a total concentration similar to that found in human milk. In this study it was concluded that ganglioside supplementation did promote

bifidobacteria while suppressing *E. coli* growth. Although the exact mechanism by which dietary gangliosides reduce fecal levels of *E. coli* has yet to be determined, interaction between specific *E. coli* strains was indicated. In addition, Drago and colleagues (27) demonstrated growth inhibition of *E. coli* and *S. enteritidis* using specific strains of *Lactobacillus* isolated from feces. Both of these studies support the fundamental hypothesis of the present study regarding the feasibility of gangliosides as probiotics. Our present knowledge is not sufficient to answer some of these important questions, but essential strides were made in ganglioside research.

Our hemagglutination-inhibition studies clearly demonstrated that gangliosides in buttermilk, yogurt, LAB, and evaporated milk inhibited the pilus-mediated adhesion of *E. coli,* $K99^+$, $CFA\text{-}I^+$, and *H. pylori* to erythrocytes. ETEC via pili (hairlike structures on their surface) have the ability to bind to the villi of the intestine and various erythrocytes. The receptors for bacteria on erythrocytes and the intestinal receptors were found to be similar. The basic premise of this study was that if bacteria were to bind to exogenous gangliosides, fewer pili would be available for adherence to the intestine, or to the *in vitro* test group of erythrocytes.

Hemagglutination inhibition was determined by comparing the amount of erythrocyte present in the supernate when gangliosides and bacteria were present in the assay to the erythrocyte count when bacteria were included alone. The increase of erythrocytes in the supernatant in the presence of gangliosides led to the assumption that gangliosides bound to the bacteria and were not available to bind to the erythrocytes, leaving them free in the supernate. It is therefore reasonable to conclude that the

adhesion of enterotoxigenic *E. coli* to the intestinal mucosa could be inhibited by the consumption of dairy products containing gangliosides.

Interpretation of the data complied, indicates that there is an important difference between the effective inhibition of gangliosides extracted from dairy products and gangliosides obtained from commercial sources (BBG, GM_3). The receptor for $K99^+$ *E. coli* has been reported to be GM_3 (84), and this specific ganglioside was more effective than the ganglioside combination present in BBG. The actual quantity of gangliosides needing to be consumed to fall within the effective range was calculated to be that contained in three to four 8-ounce yogurt containers. The negative control, $K88^+$ *E. coli*, did not agglutinate with the test erythrocytes and did not interfere with the binding that was observed to occur with gangliosides.

In an attempt to extend the research findings to another relevant enteric disease, mixed gangliosides from dairy products were tested for their ability to inhibit the adhesion of *H. pylori* to chicken erythrocytes using the hemagglutination assay. Recently, there has been considerable research into the involvement of *H. pylori* in gastric diseases (9, 10). This organism is found almost exclusively in the stomach and duodenum, and the evidence is strong that this organism is significantly involved in the etiology of duodenal and gastric inflammation and ulcers (10). Epidemiological data indicate that millions of people in the United States are chronically infected with *H. pylori.*

The ability of mixed gangliosides extracted from dairy products to inhibit the adhesion of chicken erythrocytes to *H. pylori* indicates that the hemagglutination assay could be useful in research involving this organism. The adherence of *H. pylori* to gastric epithelial cells may be critical to the maintenance of infection and ultimate pathogenicity.

H. pylori was found to contain a factor that facilitated agglutination with all types of human and many types of animal erythrocytes (53, 74). Determining whether individuals suffering from the effects of *H. pylori* respond positively to ganglioside therapy requires further research. It is reasonable, however, to assume that many of the findings involving gangliosides and enteric disease would hold true for *H. pylori.*

Screening small concentrations of extracted mixed gangliosides for growth inhibition of enteric pathogens using conventional microbiological methods is tedious and often produces conflicting results (2, 14, 99). A semi-quantitative plate assay was developed to determine the antimicrobial activity of mixed gangliosides. This double agar overlay technique, in which the top layer was a plated lawn of the test pathogen, is the basis for this assay. The double agar overlay technique has the potential to become an important tool for the rapid screening of small quantities of gangliosides. This is extremely important in light of the fact that commercial gangliosides are cost prohibitive and laboratory extraction processes are labor intensive. Using a growth-inhibiting assay, the antimicrobial activity of mixed gangliosides extracted from sweet buttermilk and cultured buttermilk, yogurt, and lactic acid bacteria (LAB) against $K88^+$ and $K99^+$ *Escherichia coli (E. coli)* was determined and met the criteria of objective three.

Mixed gangliosides were placed into shallow wells that were punched into the pathogen lawn. Zones of clearing were evident around the wells and corresponded to growth inhibition of the pathogenic bacteria. The zones were transitional, but they persisted from 2 to 16 h after incubation. In addition, samples taken from wells and incubated in a minimal medium for up to 16 h produced no bacterial growth.

Mixed gangliosides extracted from sweet buttermilk and cultured buttermilk, yogurt, and LAB were effective in inhibiting the growth of $K99^+$ *E. coli* (strain 431,0101:K30), $K88^+$ *E. coli* (strain 19K974,0141:K85:K88), and *E. coli* 0157:H7 in the range of 0.09 mM to 0.51 mM. Mixed gangliosides extracted from lactic acid bacteria were the most effective in concentrations ranging from .052 mM to .028 mM in producing zones of inhibition. Standard bovine brain gangliosides (BGG) (Sigma Chemical, St. Louis) were the least effective, with a concentration of 8.4 mM being required to produce inhibition zones.

This technique involved placing gangliosides in shallow wells punched into pathogenic bacterial lawns. Zones of inhibition produced around the wells were subsequently measured and evaluated according to specific criteria. The results of using the double agar overlay technique indicated that milk product gangliosides were effective in inhibiting the growth of $K99^+$ *E. coli,* $K88^+$ *E. coli,* and *E. coli* 0157:H7. The effects produced by the gangliosides were bacteriostatic in nature because the zones were no longer observed after 16 h.

Cultured dairy products have long been regarded as beneficial in treating enteric diseases such as diarrhea. The results from this research will contribute significantly to understanding the beneficial effects of consuming dairy products. In addition, this research has the potential to increase the understanding of ganglioside interactions in the prevention of enteric diseases in humans, especially infants, as well as in young livestock.

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