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AGGLUTINATION BEHAVIOR OF LACTOCOCCI AS DETERMINED BY ENZYME LINKED IMMUNOSORBENT ASSAY AND LASER SCANNING MICROSCOPY

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By

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ABSTRACT

AGGLUTINATION BEHAVIOR OF LACTOCOCCI AS DETERMINED BY ENZYME LINKED IMMUNOSORBENT ASSAY AND LASER SCANNING MICROSCOPY

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An ELISA was developed as a rapid method to screen lactococci for agglutination behavior and to determine antibody stability. Bovine antibodies were isolated by ammonium sulfate precipitation of colostrum secretions. The screening ELISA results suggested that IgA plays a minor role in agglutination, whereas the role of IgG might be understated in the current literature. The antibodies were then either heat treated at 71, 75, 80 or 85°C, for 1 to 90 minutes at 5 minute intervals or enzyme treated at concentrations of 0 U/nl to 135.6 U/nl of papain. Although heating to 71°C for 45 minutes was sufficient for pasteurization, effective reduction of the immunogenicity of the milk antibodies that cause agglutination as determined by the ELISA was not demonstrated. Increasing the pasteurization temperature to 75 or 80°C for 45 minutes caused decreases ($P<0.05$) in IgM, IgG and IgA antibody binding to lactococci. ELISA results indicate that papain represents a method to cleave antibodies and prevent agglutination. Laser scanning microscopic analysis using immunofluorescence confirmed the results of the ELISA for the screening, heat and enzyme procedures.

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INTRODUCTION

The occurrence of bacteriophage, antibiotic residues, sanitizers, cleaning agents and agglutination are all problems associated with the manufacture of Cottage cheese (Ryan, 1985). Agglutination is increasingly being recognized for its effects on starter performance, however some Cottage cheese manufacture's still do not fully appreciate the magnitude of the agglutination problem (Hicks and Hamzah, 1992). Therefore agglutination represents an unrecognized revenue loss to the dairy industry.

Agglutination is the result of an antibody-antigen reaction (Salih and Sandine, 1984), in which antibodies naturally present in milk bind to the surface cell receptors of bacteria. The antibodies being multivalent, join the bacteria together into large chains and clumps. The clumps grow in size until a critical mass is reached at which time the bacteria and casein precipitates to the bottom of the vat, producing yield and quality problems during cheese manufacture. Agglutination problems were first recognized in cheeses which use long incubation periods. However, Salih and Sandine (1984) observed the agglutination problem as soon as one hour after inoculation; and it has been recently suggested that agglutination may be a problem in other cultured dairy products that employ lactococci (Hicks and Milton, 1990; Ustunol and Hicks, 1994).

Many of the methods suggested to prevent the agglutination of starter bacteria have not been entirely successful or practical. Thus, there is a need for further inquiry into developing preventive measures for this problem. In light of recent advances in immunological techniques, the problem of immunoglobulin-bacterial interactions during fermentation's can be directly studied, with the ultimate goal of developing better preventative measures. The objectives of this study were: 1) To develop a modified Enzyme Linked Immunosorbent Assay (ELISA) to screen cultures for their agglutination characteristics; 2) Investigate the stability of bovine immunoglobulin to heat or enzymes using an ELISA; 3) Verify the ELISA using Laser Scanning Microscopy (LSM); and 4)Verify the stability ELISA results in a skim milk system.

1. Literature review

1.1. Lactic acid bacteria as starter cultures

Lactic acid bacteria have multiple functions when used as starter organisms for dairy products. The lactics are gram positive rods or cocci, which are nonmotile, nonpigmented, catalase negative, and facultative anaerobes (Axelsson, 1993). These bacteria ferment sugars, mainly lactose, to produce lactic acid and other compounds; and play a role in protein and lipid hydrolysis which contributes to the taste and texture characteristics of dairy products.

Starter cultures for dairy products may consist of one strain (single organisms) or multiple strains (blends of defined strains) or traditional mixed strains (undefined numbers and ratios of strains) of several organisms. These cultures are used with or without rotation depending on the manufacturer characteristics (Mayra-Makinen and Bigret, 1993). The pathway used to ferment lactose and produce lactic acid represents a distinguishing feature for classification of lactic acid bacteria. Using the Embden-Meyerhoff pathway, the homofermenters convert glucose $\rightarrow$ 2 pyruvate $\rightarrow$ 2 lactate. Whereas, the heterofermenters convert glucose $\rightarrow$ gluconic acid $\rightarrow$ CO_2 and pentose which is then converted to ethanol + lactic acid using the Hexose-Monophosphate pathway (Salminen and Wright, 1993). The main difference is that the homofermenters produce lactic acid in a 2:1 ratio when

compared to the heterofermenters. Use of homofermenter starter cultures are desirable in the production of many fermented foods, especially dairy products such as cheese where lactic acid is the desired product of the fermentation. The dairy industry relies heavily on the use of lactic acid bacteria as starter cultures. Some products include but are not limited to cheese, yogurt, sour cream, cultured milk, and cultured butter. In fact lactic acid bacteria can be used to produce over 1000 different products each with its own texture and flavor characteristics (Mayra-Makinen and Bigret, 1993).

A major concern for dairy manufacturers has been bacteriophage, which has been the main cause of starter failure (Lawrence, 1978). These bacterial viruses are strain and species specific, in that they can only attach and inject genetic material into those bacteria which have the specific attachment site. The phage insert their genetic material into the DNA of the bacteria. After replication and the production of viral proteins the cells lyse and multiple phage are released to infect other cells (Kuby, 1992). For this reason a great deal of research has been conducted in methods to inhibit phage infection and the identification of phage-resistant cultures. Other agents which cause batch failure include improperly used cleaning agents, sanitizers, as well as, antibiotics which kill the starter culture before it completes the fermentation. Another factor in starter culture performance is the interaction between immunoglobulins naturally present in the milk or media and the

starter culture. This interaction, known as agglutination, is increasingly being recognized for its effect on dairy starter cultures.

1.2. Bovine milk immunoglobulins.

The globular proteins known as immunoglobulins are the first line of defense against bacteria and viruses (Kuby, 1992). Some strains of lactococci have been known to auto agglutinate, however antibody titers (Ellerman, 1975; Emmons et al., 1966) and the frequency that the antigenic determinant is expressed on the cell surface are thought to be the major factors causing agglutination (Scheuble et al., 1989). IgA, IgG, and IgM are the three main classes of immunoglobulins identified in bovine milk, and colostrum (Butler, 1983).

IgG is the most abundant immunoglobulin found in bovine milk. IgG is found as a monomer in milk secretions and has a molecular weight of 150,000. IgG consists of two heavy chain and two light chain components (Kuby, 1992). Interchain disulfide bonds hold the heavy and light chains together forming a "Y" shape. The monomer consists of two fragments, the tail or Fc region and the arms or Fab fragments. The IgG immunoglobulin class consists of two subclasses of antibodies IgG_1 and IgG_2. IgG_1 is the major antibody found in colostrum representing as much as 80% of the whey protein component of colostrum, whereas IgG_2 is found at a level 40 times lower (Mach and Pahud, 1971). Although the amount of total antibodies is reduced in normal milk when compared to colostrum,

IgG_1 is still the predominant antibody secreted by the mammary gland (Butler, 1983). The immunoglobulin IgA is found as dimer with a disulfide bond holding two monomers together. This type of configuration allows for four possible antigen binding sites instead of the normal two found with a single monomer. The dimeric form of IgA gives it better access to antigens increasing its agglutination ability. Bovine secretions contain very large amounts of secretory IgA (SIgA). The secretory piece transports the immunoglobulin across the mucous membrane and is thought to provide protection from proteolytic enzymes. In the past, researchers have not included IgA as a contributing factor in the agglutination behavior of lactococci because of its relatively low quantities in bovine milk. In spite of the low levels, IgA will bind lactic cultures and could be a hidden factor during agglutination. IgM is found in a pentamer configuration consisting of five, four chain polymers which are linked together through disulfide bonds on the Fc portion of the immunoglobulin. This type of configuration allows for ten possible binding sites and is the reason that IgM remains the most effective agglutinating antibody (Rose et al, 1964).

In cows milk, immunoglobulins represent 0.08% of total milk protein and 3% of milk whey (Mach and Pahud, 1971). Colostrum, which is the first milk secreted after calving, contains eighty times more IgG and IgM than normal milk (Salih and Sandine, 1984). Milk contains 0.7 mg/ml of bovine IgG, however in colostrum IgG levels can reach 100 mg/ml, with levels generally returning to normal within a week

of calving (Butler, 1983). The large supply of antibodies in colostrum provides passive immunity to the newborn because the fetal calf does not acquire immunity *in utero* (Brambel, 1970). Emmons et al. (1963) reported that immunoglobulins are always present in bulk milk and it is suggested that the agglutination problem may also be partially due to contamination of bulk milk with colostrum. In Canada, as in many countries, colostrum is not permitted in the milk supply. However, Zawistowski and Mackinnon (1993) found that 89% of the samples tested in Canada were contaminated with colostrum.

In 1976, Kanno et al. isolated crude "euglobulin" fraction of milk by gel filtration and tested the agglutination activity of the different fractions against *Lactococcus lactis* ssp. *cremoris* strain HP. Of the three fractions, IgM was shown as the main factor causing agglutination. They also determined that the activity of agglutination was maximized in the pH range of 6.5 to 8.5. Salih and Sandine (1984) showed, using fluorescent techniques, that IgG bound more frequently than IgM. Although IgG is found in greater quantities, IgM with its pentameric structure is thought to be the primary agglutinating factor (Kanno et al., 1976).

1.3. Starter culture agglutination

Acid coagulated cheeses are manufactured by precipitation of the milk proteins as the pH is lowered (by acidification) until the isoelectric point is reached. The average isoelectric point for casein micelles is approximately 4.6 at which time

the casein micelle unfolds and a protein network is formed trapping fat and moisture in the matrix. Acidification can occur in two ways, the first is direct addition in which food grade acid is added directly to the vat. The second method involves the use of starter cultures in which bacteria produce the acid that ultimately leads to coagulation. Any factor that inhibits culture performance can have a consequence on acid production, thus coagulation, effecting both the yield and the quality of the product.

The ability of certain substances naturally present in milk to interfere with coagulation was first shown by Stocking (1904). Agglutination is the result of an antigen-antibody reaction as shown by Salih and Sandine (1984). This problem is the result of agglutinins (immunoglobulins) naturally present in milk binding to the surface cell receptors or epitope's of the starter bacteria. The antibodies being multivalent can bind to multiple bacteria causing them to aggregate and forming chains or clumps. The bacteria in these clumps continue to produce acid which precipitates the casein's around the bacteria and adds to the weight of the complex. When a critical mass is reached, the cells begin to sediment, (Milton et al., 1990) causing uneven distribution of the bacteria in the vat and thus uneven acid leading to yield and quality problems in production (Milton et al., 1990). This was also observed by Weeks et al. (1994) who reported the same settling of bacteria during heavy coagulum formation.

A closely related phenomena to agglutination is aggregation which occurs for conjugation purposes. The aggregation of cells greatly enhances the ability for effective transfer of genetic information. A study by Wang et al. (1994) showed that the efficiency of genetic transfer was 10^2 - 10^5 times higher for bacterial cells that are able to self aggregate. Upon PAGE analysis it was determined that aggregation positive cells contained a unique protein 125 kDa in size. Under these circumstances cell aggregation is a positive adaptation for lactic acid bacteria. Unlike cell aggregation, agglutination, however, is a negative aspect which results in a decrease in acid production and loss revenues due to decrease quality and yield.

Morice et al. (1991) found that *Streptococcus thermophilus* will grow in shorter chains in the presence of cations such as Mn^{2+}, Ca^{2+} Mg^{2+}, Fe^{2+}, and Zn^{2+}. They determined that sodium had a shorting effect in 17 out of 20 strains of *Streptococcus thermophilus* with best reductions occurring with sodium concentrations over 80 mM. They also concluded that the anion does not play a role in the dechaining activity of sodium salts.

Weeks et al. (1994) reported the occurrence of a layer of heavy coagulum containing high total solids on the base of commercial lactic casein silos. They termed this phenomenon heavy coagulum formation and concluded that this occurrence was similar to that proposed by Emmons et al. (1966) for minor sludge formation in Cottage cheese. Commercial lactic casein manufacture differs from Cottage cheese manufacture by the size of the coagulation vessel and the length of

incubation. The incubation for Cottage cheese is roughly 5-6 hours, which is considerably less than that of the 14-16 hours needed for lactic casein manufacture. Yield loss is not the major concern in heavy coagulum formation due to an efficient fines recovery system. However, the variation produced creates difficulty during processing and requires a blending step. Therefore, the elimination of heavy coagulum formation as agglutination would greatly benefit the dairy industry.

1.4. Factors affecting agglutination

Emmons et al. (1963) observed that immunoglobulins are always present in bulk milk. Bovine milk contaminated with colostrum favors agglutination; but milk that has been homogenized or pasteurized at higher than normal temperatures, reduce agglutination (Emmons et al., 1966). The degree of agglutination is dependent upon the strain of bacteria and the agglutination titer (Scheuble et al., 1989). The variability between strains is the result of the number of epitopes found on the surface of the bacteria. The strains of bacteria that contain more epitopes will be more prone to binding antibodies and thus more to agglutination. However, Emmons et al. (1966) suggested that it was the antibody titer which is most important in determining the severity of agglutination.

Emmons et al. (1966) also reported that chain length increased the extent of settling. Scheuble et al. (1989) observed no significant difference based on chain length in cultures that agglutinate severely, compared to those that do not

agglutinate at all. Shorter chains of bacteria decreases the consequences of agglutination as they will lack the critical mass necessary to sediment and will be more evenly distributed in the vat. The cultures that grow in longer chains upon agglutination will reach the critical mass quicker and thus the extent of settling will occur sooner and to a greater extent. Ibrahim (1995), reported that the proteolytic activity of starter cultures might be a clue into the agglutination behavior of bacteria. He reported that the proteolytic activity of agglutination sensitive cultures was lower than that of agglutination resistant cultures. The amount of agitation that occurs may have a pronounced effect upon agglutination severity. Prolonged agitation was once thought to increased acid production and decreased culture agglutination in skim milk (Gillies, 1959). However, Emmons et al. (1966) documented a decrease in acid production and an increase in culture agglutination upon prolonged agitation. Gosling et al. (1995a) recently reported that agitation during coagulation could either increase or decrease heavy coagulum formation depending on the timing and geometry of agitation.

Antibodies are naturally present in whey based media, which are typically used to grow lactic starter cultures for vat inoculation. For that reason, the bacteria could agglutinate in the bulk starter media before being added to the vat. In severe cases, batch failure can occur if the lactococci has already agglutinated in the media before addition to the vat.

1.5. Methods to inhibit culture agglutination

Chemical denaturants such as 2-mercaptoethanol can denature antibodies by dissociating the disulfide bonds allowing for separation of the heavy and light chains. Kanno et al. (1976) saw 76%-86% reduction in agglutination activity upon treatment with 0.2 M 2-mercaptoethanol for three hours at room temperature or one hour at 30°C. Surfactants have been used to decrease chain formation (Hicks and O'Leary, 1986). It was once believed that lecithin may hinder attachment of antibodies by changing or coating epitopes on the cell surface (Stollerman and Ekstedt, 1957). Recently, Milton et al. (1990) determined by cell counts that increases in colony forming units occurred when low concentrations of lecithin were used but then a decrease in agglutination as concentrations were increased. They observed the greatest increase, 0.3 $\log_{10}$, when 0.5% lecithin treatment was used and than a decrease in bacteria counts as the treatment levels were increased to 1% and 2%. The use of lecithin could increase agglutination by facilitating chain fragmentation (Milton et al., 1990).

Enzyme cleavage also represents a way to destroy antibodies. The enzyme papain is a sulfhydryl protease isolated from *Carica papaya*, and has a molecular weight of 23,000. After brief exposure to the antibody, the enzyme papain will cleave the most susceptible bonds. Papain has nonspecific activity and will digest the entire protein if given proper time. The most susceptible bonds on an IgG immunoglobulin are on the heavy chain below the interchain disulfide bonds. After

digestion, this leaves two Fab fragments which may still bind to the antigen and a Fc fragment (Kuby, 1992). Ustunol and Hicks (1994) suggested that Fab fragments from papain digestion will bind to the antigenic binding sites on the bacteria and prevent further agglutination interactions by blocking fully intact immunoglobulins present either in the media or milk. In their study, antibodies from whey fractions were digested with papain during ultrafiltration and diafiltration to isolate those antibody fragments with a molecular weight less than 10,000 Da. These fragments were then freeze dried and added to an internal pH control buffer salt mixture. Commercial cultures OS, M30, and M37 when grown in this reconstituted media show pH differential reductions of 40, 55 and 72% for the cultures OS, M30 and M37 respectively. The enzyme pepsin will also cleave antibodies, however unlike papain, pepsin cleaves the bonds just below the interchain disulfide bonds. On an IgG molecule pepsin produces one main fragment called $F(ab)_2$ fragment and many small fragments that composed the Fc fragment. The $F(ab)_2$ fragment consists of the two Fab fragments joined by interchain disulfide bonds. The joining still allows it the capability of precipitating the antigens (Kuby, 1992).

The use of heat to inactivate antibodies was first shown by Emmons et al. (1966). They reported eliminating the agglutination activity in milk by heating at 71°C for 30 minutes. Dominguez et al. (1997) determined that no loss in antigen binding activity of IgG occurred after 60 minutes at 65°C. Lindstrom et al. (1994) concluded that thermal treatment of immunoglobulin will cause the antibodies to

unfold and aggregate. They reported that the unfolding of bovine antibodies due to heat is irreversible and not very dependent on pH as determined by differential scanning calorimetry. De Wit and Klarenbeek (1984) reported that IgG unfolds at 72°C in 0.7 M phosphate buffer, with values extrapolated to a heating rate of 21.4° K/min. Fukumoto et al. (1994) who using radial immunodiffusion reported D values of 329 minutes at 70°C, 38.8 minutes at 74°C, and 4.3 minutes at 80°C. Dominguez et al., (1997) reported that at 72°C it took only 1387 seconds for a 90% reduction in the binding activity of IgG and suggested that the ability of IgG to bind antigens after heat treatment probably has been overestimated in the literature. Li-Chan et al. (1995) reported slightly higher D values for IgG than Dominguez et al., (1997). Li-Chan et al. (1995) concluded that a substantial portion of the antibody activity is retained in milk after commercial processing. Lindstrom et al. (1994) reported that immunoglobulins are among the most heat stable of the whey proteins and concluded that a temperature of 80.9 $\pm$ 0.04°C was needed for the thermal unfolding of the immunoglobulin fraction of whey. This was consistent with Ruegg et al., (1977) who determined that the thermal unfolding of immunoglobulins was at 81°C in simulated milk ultrafiltrate. Later, De Wit et. al. (1983) reported that the immunoglobulin fraction denatured at 79°C.

Homogenization of bulk starter culture has been investigated as a method to inhibit culture agglutination by Milton et al. (1990) and Russell-Campbell and Hicks (1992). A homogenization pressure of 246 Kg/cm^2 was sufficient to inhibit

agglutination in milk by mechanically breaking apart the chains of starter cultures and reducing the critical mass. The homogenization of milk also has been investigated, and it is believed that the homogenization of skim milk causes the skim milk membrane to conform and expose antibody binding sites allowing antibodies to bind the skim milk membrane instead of the lactic starter (Russell-Campbell and Hicks, 1992). Russell-Campbell and Hicks (1992) determined that as the homogenization pressure is increased, more antibody binding sites become available on the skim milk membrane, leading to a decrease in agglutination tendencies of the culture. For this reason, they recommended that skim milk used for Cottage cheese manufacture be homogenized at a pressure of at least 176 kg/cm^2 to reduce agglutination. However, the use of homogenization to prevent agglutination increases equipment expenditures, the labor requirements, and ultimately the cost of production.

Similar to homogenization, the use of colloid milling was investigated by Gosling et al. (1995a) in which a colloid mill was used to reduce heavy coagulum formation. They reported that homogenization is an unlikely solution for heavy coagulum formation due to high capital costs and low throughput of homogenizers. Colloid mills are cheaper and have a greater throughput. When used at the proper time, colloid mills caused complete elimination of heavy coagulum. The main drawback to this technology is that the milling step needs to take place at 11-12 hours to reduce heavy coagulum formation at which point the incubation continues.

The timing of this milling step, however, may create logistical problems for manufacturers.

1.6. Defects associated with bacterial agglutination

Agglutination causes two major defects in cultured dairy products. The first occurs if the clumps of bacteria are suspended in the cheese milk as coagulation occurs, thus trapping the bacterial casein complex before it sediments to the bottom of the vat. This results in a grainy defect in the curd upon cutting (Emmons et al., 1963; Emmons et al., 1966). The second defect is more severe and occurs when the bacterial complexes aggregate at the bottom of the vat to produce a "sludge layer." In its severest form, agglutination will prevent milk from coagulating, leaving the vat unusable. The sediment or sludge layer may have a slight brown color which indicates that casein from the skim milk was precipitated by the complex of cells. Even minor sludge formation associated with agglutination may be routinely responsible for yield losses of 4-8% (Gradison et al., 1986). Slow uneven acid production, grainy curd, sediment formation, along with an easily shattered curd after cutting, produce inconsistent and poor quality cheese (Emmons et al., 1966). These defects result in decreased profits for dairy manufacturers by limiting yield and quality.

1.7. Agglutinating detection and screening procedures

Under optimum conditions, lactic starter culture and thus acid production should be uniform throughout a cheese vat during fermentation. Bacterial agglutination is characterized by uneven acid production where the pH at the bottom of the vat is typically lower than the pH at the top of the vat due to the settling of starter bacteria. For this reason, the pH differential between the top and bottom pH has been used to determine bacterial agglutination by a number of researchers (Emmons et. al., 1963; Emmons et al., 1966; Salih and Sandine, 1984; Milton et al., 1990 and Ustunol and Hicks, 1994). Typically, the greater the pH differential, the greater the severity of agglutination. Milton et al. (1990) used a pH differential greater than 0.2 as an indicator of culture agglutination, whereas Hicks and Ibrahim (1992) used 0.09 units and Ibrahim (1995) reported 0.1 units to indicate culture agglutination. Other researchers have reported that pH measurements were an unreliable method to determine or monitor heavy coagulum formation (Gosling et al., 1995b).

Due to the settling of bacteria, microbial enumeration can also be used as an indicator of agglutination (Emmons et al., 1966; Milton et al., 1990). If culture agglutination has occurred, the numbers of bacterial cells will be greater on the bottom of the vat. For that reason, standard plate count or direct microscopic examination has been used as a simple, rapid test for bacterial agglutination (Ibrahim, 1995). This is based on the idea that agglutination sensitive cultures

appear in long chains and clumps, whereas agglutination resistant cultures are found in smaller chains with little or no clumping.

Another characteristic of severe agglutination would be the sludge or sediment found on the bottom of the vat. By measuring the depth of the sediment, one can obtain a determination of agglutination severity. This can be performed by direct visual measurement or using a pH meter. Since the sediment contains a lower pH due to the high concentration of lactic bacteria, the sediment layer can be measured by the pH drop that occurs when the electrode is inserted into the sediment (Hicks and Hamzah, 1992). Another method to detect agglutination is the comparison of top and bottom total solids. As bacteria sediment and deposit on the bottom of the cheese vat, total solids will increase. When agglutination is occurring, the total solids from the bottom of the vat will be greater than the total solids from the top. For commercial Cottage cheese vats, Hicks et al. (1989) determined that total solids was a more effective and sensitive method to determine culture agglutination than either pH differentials or bacterial enumeration. They concluded that a difference of 0.07% between top and bottom total solids can be used as an indicator of slight agglutination. This was later supported by Gosling et al. (1995b) who reported that measuring total solids in a suitable coagulation tube is an effective method to screen cultures for heavy coagulum formation. They reported that this method is more quantitative and, thus, more objective than other screening tests.

Agglutination can also be detected using fluorescent techniques. Salih and Sandine (1984) used anti-IgM, and anti-IgG with a fluorescein tag to assess agglutination. Scheuble et al., (1989) also demonstrated fluorescent techniques by staining lactic cells with laser grade rhodamine 123, which is a fluorescent cationic probe taken up selectively by bacterial cells. They incubated the cells with colostral whey proteins and viewed them under an epifluorescent microscope and confirmed that actual cells were involved in the agglutination reaction.

The methods used to determine which bacterial strains will agglutinate have not been entirely successful. Most commercial starters consist of mixed strains, which may contain both agglutination resistant and agglutination susceptible bacteria. Auclair and Vassel (1963) found that many cultures, although classified as resistant, may contain agglutination sensitive strains which become dominant upon extended subculturing in autoclaved milk. The scenario also exists for bacterial agglutination to play a larger role in the quality and yield of a product if the resistant strain is eliminated by phage.

Several researchers (Emmons et al., 1965; Salih and Sandine, 1984; and Ellerman, 1975) developed procedures to test strains for agglutination resistance or sensitivity. Emmons and Ellerman's tests were not completely effective and they were time consuming. Emmons et al. (1965) developed a test that used a stereomicroscope to visualize agglutination; however, the endpoint determination for their test is rather subjective. Ellerman's test involved using microtiter plates, in

which bacteria and whey samples were incubated for 18-20 hours in round bottom wells. Those cultures that did not agglutinate would settle to the bottom of the wells and form a pellet, and conversely cells that did agglutinate would spread out and form a thin layer, thus the pellet would be larger than the nonagglutinating culture. This test relies on the operator to determine the endpoints, making it a subjective test as well.

A rose bengal stain assay has been developed for the rapid screening of *Brucella* agglutinins. Salih and Sandine (1984) used this information to develop a procedure to screen for culture agglutination. The procedure requires the researcher to harvest the cells using a centrifuge and then heat inactivate in a 60°C water bath for one hour. After centrifugation and dilution, the cells are stained with rose bengal stain. The procedure requires the researcher to place a drop of stained cells and a drop of whey on a Brewer's diagnostic card. The cells and whey are then mixed together with a sterile toothpick and the plate is shaken for 4 minutes to allow for maximum agglutination. The agglutination of cells is determined by looking at the reaction of stained cells and whey. A positive result has large, clear aggregate of clumping cells and negative result has no clumping. This procedure was then modified by Scheuble et al. (1989) to decrease antigen preparation time. The problems associated with this assay are test time, accuracy, reliability, subjectivity in determining the endpoints, and application to a plant setting. In spite of these deficiencies, Thunell et al. (1984) used such test routinely in Cottage

cheese plants to identify and replace agglutination sensitive cultures with agglutination resistant cultures and effectively reduced agglutination.

Screening procedures developed to date may not be entirely effective or practical. This is due to the long time requirements involved for the assay, and the subjectivity in interpreting the results. For these reasons, the development of a rapid, nonsubjective test is still needed. The use of ELISA's in the food industry has received much attention in recent history. The ELISA is an objective, sensitive, rapid, simple test that does not require extensive training as many other tests do.

The elimination of agglutination could save millions of dollars in the Cottage cheese industry. Gradison et al. (1986) suggested that minor sludge formation might be routinely responsible for yield losses of 4-8% This represents a considerable amount when results for 1996 showed that over 360 million lbs of creamed Cottage cheese and 329 million lbs of low fat Cottage cheese were produced (National Agricultural Statistics Service, 1996). A conservative estimate of a 1% increase in Cottage cheese yield would approximately represent an additional 3,600,000 lbs of creamed, and 3,290,000 lbs of low fat Cottage cheese annually. The increase in yield and quality would represent about $7.5 million to the Cottage industry alone. Researchers have suggested that agglutination may also be a problem in other dairy products that use lactococci as starter cultures (Milton, 1990; Ustunol and Hicks, 1994; Weeks et al., 1994).

2. Materials and Methods

2.1. Cultures

Frozen multiple strain commercial cultures of *Lactococcus lactis* subs. *lactis* and *Lactococcus lactis* subsp. *cremoris* (M30, M27 and SG1) were provided by Rhone-Poulenc (Marschall Div., Madison, WI). Single strain cultures WWA, M52, B62, E72, 478, and 874 were obtained from Chris Hansen's Laboratory (Milwaukee, WI) and were chosen because of their different agglutination characteristics. All cultures were stored at -80°C until use.

Both single and multiple strain cultures were screened using the method of Hicks and Ibrahim (1992). Briefly this procedure involves inoculating the cultures at a 5% (v/v) level into reconstituted nonfat dry milk (NDM)(10% (w/v) solids autoclaved 10 min at 121°C and stored at 4°C until use). The inoculated NDM was incubated for 18 h at room temperature(~ 23°C). Each culture was then inoculated into 50 g reconstituted NDM as prepared above and incubated for 18 h at room temperature (~23°C). The 50 g of culture were then added at a 5% (w/v) level to 950 g of pasteurized (63°C for 30 min) skim milk in sterile (15 min at 121°C)1000 ml graduated cylinders. The cylinders were incubated in a 30°C water bath for 5 h. Agglutination was determined visually at the end of the 5 h incubation. If sediment was observed at the bottom of the cylinder, it was scored as positive (+), whereas

cultures with no visible sediment were scored negative (-). This procedure was replicated 3 times with cultures having three negative replications (-/-/-) classified as resistant, three positive (+/+/+) classified as sensitive, and 1 (+/-/-) or 2 (+/+/-) positives classified as having moderate agglutination behavior.

2.2. Colostral antibody preparation

Bovine colostrum was obtained from the Michigan State University Dairy Farm. Antibodies were purified by ammonium sulfate precipitation of colostrum. A 33% solution of ammonium sulfate was added to colostrum, and centrifuged (3,000 x g) for 20 min. The isolate was redissolved in an equal volume of 0.01M phosphate buffered saline (PBS; 32.0 g NaCl, 4.99 g Na_2HPO_4, 1.029 g Na_2PO_4, in 4 Liters of DH_2O, pH 7.4) The centrifugation process was then repeated 2x. The concentrate was then dialyzed for 3 days against PBS at 4°C with daily changes of PBS. The antibodies were aliquoted and frozen at -80°C.

2.3. ELISA procedures

The cultures were propagated overnight at room temperature (~23°C) in Ellikers broth (Difco, Detroit, MI). The cultures were then heat killed in boiling water for 2.5 h. The cells were centrifuged (3,000 x g) for 1 min. The pellet was resuspended in 1ml of sterile PBS. The washing was repeated three times in PBS, with the final pellet being diluted to give an optical density reading of 14%

transmittance at 590 nm (Spectronic 1001, Spectronics Instruments Inc., Rochester, NY) in 0.1M carbonate-bicarbonate coating buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 0.2 g NaN_3, in 1 liter of DH_2O, pH 9.6). Immunolon 4 plates (Dynatech Laboratories Inc., Chantilly, VA) were coated with 50 µl per well of either culture or control, and wrapped in parafilm, aluminum foil, and incubated overnight at 4°C. The plates were then washed 3 times in 0.02% PBS Tween-20 (PBS-T) (Sigma Chemical Co., St. Louis, MO), and then blocked with 300 µl of 1.0% Gelatin type A from Porcine skin (Sigma) for 30 min and washed 4 times in 0.02% PBS-T. The plates were incubated at 37°C for 1 h with 50 µl of antibody sample per well, prepared as described below. After washing 5 times in 0.02% PBS-T solution, the plates were incubated for 1 h at 37°C with 100 µl of either Anti-IgG (0.25 ng), Anti-IgA (1.0 ng), or Anti-IgM (2.0 ng) (Bethyl Laboratories, Inc., Montgomery, TX) diluted in the blocking solution. After washing 6 times, peroxidase binding was determined using 2,2'Azinobis(3-ethylbenzo-thiazoline-sulfonic Acid) (ABTS)(Sigma) (ABTS)-H_2O_2 substrate as described by Pestka et al. (1982). Absorbence at 405 nm was read using a ELISA plate reader (Molecular Probes, Inc., Eugene, OR.) using Softmax software. A larger optical density was the result of more antibodies binding to lactococci whereas a lower optical density represented fewer antibodies bound. The ELISA results for the stability of bovine antibodies IgM, IgG, and IgA were converted to percents by the following formula: % control OD = 100 -[100(OD control - OD

treatment) / OD control]. The zero time on the heat treatment graphs was when the media was brought to temperature, with all control bindings being 100%.

2.3.1. Sample preparation for ELISA

2.3.1.1. Effect of heat on stability of bovine milk immunoglobulins and their ability to bind lactococci

Insure media (Galloway West Co., Fond Du Lac, WI) buffer salts was reconstituted according to the manufacturer's directions. Colostral antibodies were diluted 1:5 (v/v) into Insure media for a final volume of 50 ml and heat treated in stirred water baths. Samples were taken at 0, 1, 5 min and every 5 min interval thereafter up to 90 min at 71°C and 75°C. The time intervals were the same for 80 and 85°C, except the last samples at this temperature were taken at 60 min and 30 min, respectively. Samples were immediately placed on ice and stored at 4°C until use in the ELISA the next day. The ELISA to determine heat stability of IgM, IgG, and IgA and their ability to bind lactococci was conducted as described previously in Section 2.3.

2.3.1.2. Effect of papain and pepsin on stability of bovine milk immunoglobulins and their ability to bind lactococci

Insure media (Galloway West Co.) buffer salts were reconstituted according to the manufacturer's directions. Colostral antibodies were diluted 1:5 (v/v) into Insure media for a final volume of 50 ml and treated either with papain (Sigma) or

pepsin (Sigma). Five concentrations of enzyme solution 0%, 5%, 10%, 15%, 20%, and 25% (v/v) were chosen from preliminary studies. These concentrations when converted to enzyme activity units (U) which correspond to 0 U/nl, 27.1 U/nl, 54.3 U/nl, 81.3 U/nl, 108 U/nl, 135.6 U/nl for papain and to 0 U/nl, 375 U/nl, 750 U/nl, 1,100 U/nl, 1,500 U/nl and 1,875 U/nl for pepsin. The concentrations were acquired by adding a saturated solution of papain (0.65g in deionized water) or pepsin (1.50 g in 3 ml deionized water) to the 1:5 antibody/media mixture. The antibody/media enzyme solution was incubated at 37°C for 2 h (1 h in a oven, and 1 h as the sample in the ELISA described in Section 2.3). After incubation, the enzyme and any unattached antibodies were washed out of the ELISA plate during the washing procedure. The ELISA to determine enzyme stability of IgM, IgG, and IgA and their ability to bind lactococci was conducted as described previously in Section 2.3.

2.3.1.3. Screening culture for agglutination behavior

Colostral antibodies were diluted 1:5 (v/v) into 1.0% gelatin blocking solution for a final volume of 50 ml. The ELISA to determine the amount of IgM, IgG, and IgA able to bind lactococci was conducted as described previously in Section 2.3.

2.4. Laser scanning microscopy

The single and multiple strain lactococci were grown in Insure media (Galloway West Co.) overnight until a pH of 4.6 to 5.2 was reached. 240 µl of culture were centrifuged (3,000 x g) for 1 min. The pellet was resuspended in 160 µl

and then washed and resuspended in 80 μl, washed and resuspended in 2 μl of PBS and allowed to air dry on a slide. The culture smear was circled with a Peroxidase-Antiperoxidase-Peroxidase (PAP) pen (Research Products International Corp., Mt. Prospect, IL) to create a well. Fixing agent (ethanol, chloroform, formalin 6:3:1 ratio) was added to the slides. After 7 min the slides were rinsed with 95% ethanol. After rinsing with distilled H_2O, the slides were heat fixed by passing through an open flame. Previously prepared colostrum antibodies (50 μl) as described in Section 2.3.1., were added and allowed to react for 15 min at room temperature (~23°C). Excess antibodies were then washed off the slide with PBS. Fluorescein conjugated antibodies were diluted in PBS with either a 1:50 (v/v) ratio for anti-IgG fluorescein (2.0 ng/μl), 1:15 (v/v) anti-IgM fluorescein (5.5 ng/μl) or 1:15 (v/v) anti-IgA fluorescein (6.7 ng/μl) antibodies (Bethyl Laboratories, Inc., Montgomery, TX.), and incubated another 15 min on the slide. The slides were washed with PBS, deionized H_2O and air dried. After applying mounting fluid (Difco) and a cover slip, the slide was viewed using a Zeiss 10 Laser Scanning Confocal Microscope (Carl Zeiss Inc., Jena, Germany). The 40x oil objective with a zoom of 100x was used with the contrast set at 820 units for the transmitted images and 353 units for the fluorescent images. Brightness was set at 180 units and 457 units, respectively on the computer monitor for the transmittance and the fluorescent pictures. The LSM pictures presented are split screen images in which the right side is a phase contrast image and the left side is a fluorescent image of the same frame.

2.5. Verification of agglutination in skim milk

Skim milk was obtained from the Michigan State University Dairy Plant. Agglutination in skim milk was determined by inoculating fermented media at a 5% level (v/v) into pasteurized (63°C, 30 min.) skim milk contained in a sterile 1,000 ml graduated cylinders and incubated at 32°C for 5 h. Agglutination was monitored using three methods: 1) top and bottom pH, 2) depth of sediment and 3) total solids. Top and bottom pH measurements in skim milk were measured up to 5 h with readings taken at 1 h intervals. The top pH was measured 5 cm below the surface of the milk. Whereas the bottom pH was measured with the probe resting on the bottom of the cylinder. The pH differential was computed by subtracting the bottom pH from the top pH. A pH differential of 0.1 or greater between top and bottom was used as an indicator of agglutination. The second method to determine agglutination was depth of sediment, which was measured through the side of the graduated cylinder using a centimeter ruler. For the final method, samples of the curd were collected at the end of the 5 hr incubation period and stored at 4°C overnight for total solid determination the following day. Three gram duplicate samples were taken from 5 cm below the surface and from the bottom of the cylinder. Total solids were determined the day after sampling. Samples were dried in 100°C oven for 16 h, cooled in a desiccator until a constant weight was obtained for total solid measurements. The % total solids =[(dry weight) x 100] / wet weight

(Hicks and Hamzah, 1992). The differential was determined by subtracting the top from the bottom total solids.

2.5.1 Sample preparation for verification of agglutination in skim milk

2.5.1.1. Effect of heat on the stability of bovine milk immunoglobulins and their ability to bind lactococci

Insure media was fortified with a 1:5 ratio of antibody to buffered salt solution and then divided into aliquots of 125 ml. Samples were heat treated for 45 min at 71, 75 or 80°C in a water bath. The media was then cooled to room temperature under cold tap water and stored at 4°C for 16 h. The media was brought to room temperature prior to inoculation with the bacterial culture. Next, the cultures were incubated overnight at room temperature (~ 23°C) until inoculation into the skim milk as described previously in Section 2.5.

2.5.2.2. Effect of enzyme treatment on the stability of bovine milk immunoglobulins and their ability to bind lactococci enzyme study verification

Insure media was fortified with a 1:5 ratio of antibody to buffered salt solution solution and then divided into aliquots of 125 ml. Papain enzyme concentrations 0 U/nl, 27.1 U/nl, 54.3 U/nl, 81.3 U/nl, 108 U/nl, and 135.6 U/nl were incubated for 2 h at 37°C prior to pasteurization at 71°C for 45 min. Next the cultures were incubated overnight at room temperature (~ 23°C) until inoculation into the skim milk as described in previously in Section 2.5.

2.6. Statistical analysis

The ELISA, milk verification and screening experiments were replicated four times in a randomized block design. The data was analyzed using a one-way repeated measures Analysis of Variance with Sigma Stat Analysis System Software (Jandel Scientific, San Rafael, CA) with the exception of the enzyme ELISA study which used a nonparametric test based on ranks, for IgM and IgA stability to papain treatment when binding to M27, and IgM and IgG stability to papain treatment when binding to M30. Pepsin treatment to IgA for the cultures M27 and SG1 also used a nonparametric test because the data violated the assumptions of a parametric test. Statistical differences were computed by the Student-Newman-Keuls method, with a $P<0.05$ considered statistically significant.

Results and Discussion

3.1. Screening of cultures for agglutination behavior.

3.1.1. Culture agglutination as determined by the ELISA

The agglutination behavior of lactococci determined by the screening method of Hicks and Ibrahim, (1992) together with the suppliers reported agglutination behavior are shown in Table 1. The classification of E72 and BG2 as resistant by the method of Hicks and Ibrahim (1992) was in sharp contradiction to agglutination behavior reported by the supplier Chris Hansen. Scheuble et al. (1989) stated that the severity of agglutination for a particular strain depends on two factors: 1) the frequency with which a specific antigenic determinant is expressed on the cell surface and 2) the agglutination titer. Thus, the amount of immunoglobulin that bacteria bind could be used as an indicator of agglutination.

The agglutination behavior of bovine IgM, IgG and IgA to bind lactococci are presented in Figure 1 and Table 2. The agglutination sensitive single strain cultures M52, E72 and BG2 showed the greatest affinity ($P<0.05$) for binding IgM with M52 and E72 binding similar amounts (Table 2). The M30 culture bound less IgM than the single strain cultures M52, E72 and BG2, yet greater than the other lactococci WWA, SG1, OS, 478, 874, and M27. The two moderate agglutinating cultures 478

and 874 bound similar amounts of IgM (Table 2). Agglutination resistant WWA, OS and SG1 along with agglutination sensitive M27 showed the least affinity

Table 1. Comparison of agglutination behavior of lactococci provided by the supplier, to the screening method of Hicks and Ibrahim (1992).

Culture	Agglutination behavior provided by supplier	Hicks and Ibrahim screening Method (1992)
WWA	resistant	resistant
OS	resistant	resistant
SG1	resistant	resistant
478	minor to moderate	minor to moderate
874	minor to moderate	minor to moderate
M52	minor to moderate	minor to moderate
E72	sensitive	resistant
BG2	sensitive	resistant
M30	sensitive	sensitive
M27	sensitive	sensitive

to bind IgM (Figure 1; Table 2). With the exceptions of WWA, 478 and 874 and also E72 and M52 which bound similar amounts, all other treatments were different ($P<0.05$) from each other (Table 2). The agglutination sensitive M27 culture bound the least amount of IgM for all lactococci tested in my research with the exception of SG1.

SG1 showed the least affinity for binding IgG (Figure 1; Table 2), whereas WWA and OS bound similar amounts of IgG (Table 2) but more than SG1. E72 and BG2 showed the greatest affinity to bind IgG (Figure 1; Table 2).

Table 2. Ability of single and mixed strain lactococci to bind bovine milk immunoglobulins as determined by the ELISA.

Culture	IgM	IgG	IgA
	Optical Density Mean[1]		
WWA	0.334[a] (0.12)	0.590[a] (0.21)	0.304[a] (0.10)
OS	0.297[b] (0.11)	0.506[a] (0.22)	0.242[b] (0.07)
SG1	0.202[c] (0.07)	0.363[b] (0.15)	0.215[c] (0.08)
478	0.329[a] (0.15)	0.765[c] (0.23)	0.265[b] (0.09)
874	0.344[a] (0.14)	0.698[d] (0.10)	0.260[b] (0.09)
M52	0.712[d] (0.26)	0.757[d] (0.18)	0.682[f] (0.19)
E72	0.745[d] (0.41)	0.986[e] (0.18)	0.839[g] (0.20)
BG2	0.885[e] (0.21)	0.947[f] (0.20)	0.808[h] (0.11)
M30	0.422[f] (0.14)	0.754[d] (0.15)	0.312[a] (0.12)
M27	0.274[g] (0.09)	0.764[d] (0.13)	0.256[b] (0.11)

[1] Optical Density Mean with standard deviation in parentheses.
Means with different superscript are statistically different ($P < 0.05$) for comparisons made within the same column. Results of 4 independent treatments.

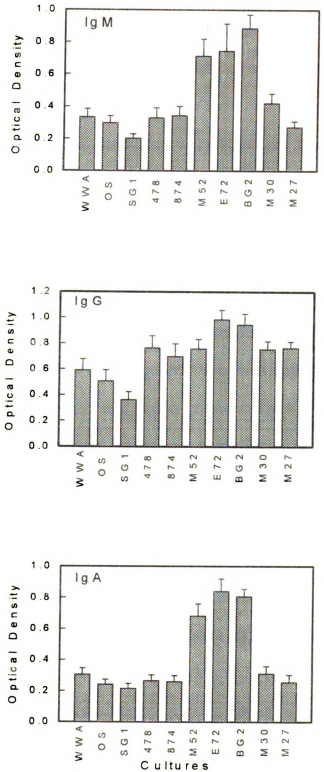

Figure 1. Ability of single and mixed strain lactococci cultures to bind IgM, IgG and IgA as determined by the ELISA.

Both the moderate agglutinating and those sensitive to agglutination showed a greater affinity to bind IgG than the agglutination resistant cultures WWA, SG1 and OS (Figure 1; Table 2).

Kanno et al. (1976) determined that IgM was the main antibody causing agglutination and IgG was a factor due to its large quantities. It was observed with the ELISA data that the single strain nonagglutinating culture WWA binds relatively more IgM and IgA than the single strain moderate agglutinating culture 478. However, 478 binds more IgG than WWA. The fact that WWA is a nonagglutinating culture that binds more IgM and IgA than the moderate agglutinating 478 suggest that IgG may play a larger role in determining culture agglutination behavior than previously expected. In this study those lactococci that agglutinated tended to bind more IgG than lactococci that did not agglutinate (Table 2). The ELISA results (Table 2) suggest that lactococci which bind high quantities of IgG will agglutinate.

M52, E72 and BG2 showed the greatest affinity to bind IgA, whereas SG1 showed the least (Figure 1; Table 2). The M30 and WWA cultures bound IgA at a lower quantity than single strain agglutinating cultures M52, BG2 and E72 (Figure 1; Table 2). The remaining cultures with different agglutination classifications bound similar amounts of IgA (Table 2). The ELISA results (Table 2) suggest the binding of IgA does not play a major factor in the agglutination of cultures since both nonagglutinating, moderate agglutinating, and agglutinating sensitive cultures bind similar quantities of IgA.

The results of the ELISA (Figure 1; Table 2) indicate that single strain agglutination sensitive cultures BG2 and E72 bind more bovine IgM, IgG and IgA than the single strain minor agglutinating cultures M52, 478 and 874. The ELISA results (Figure 1; Table 2) indicate that E72 and BG2 bind large quantities of bovine immunoglobulins which indicates that these lactococci are sensitive to agglutination, and thus supports the agglutination behavior reported by the supplier of the cultures and contradicts the screening method of Hick and Ibrahim (1995). The mixed strain agglutination cultures M30 and M27 bound more IgM, IgG and IgA than the mixed strain agglutination resistant cultures OS and SG1 with the exception of OS which bound more IgG than M27 (Table 2). For the most part the single strain cultures bound more immunoglobulins than mixed strain cultures with the same agglutination description (i.e., resistant, moderate, sensitive). This is most likely due to the presence of agglutination resistant strains within an agglutination sensitive mixed strain culture.

3.1.2. Agglutination behavior as determined by laser scanning microscopy

Microscopic analysis of the cultures with LSM using immunofluoresence to detect IgG confirmed the ELISA results (Table 2). The ELISA results (Table 2) determined that lactococci bound more IgG than IgM or IgA and for that reason IgG was chosen as the fluorescent staining agent. IgG was also previously used in fluorescent work done by Salih and Sandine, (1984). Agglutination sensitive

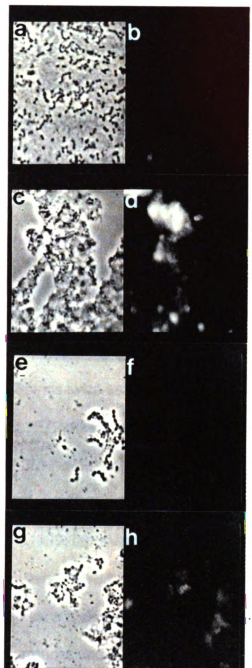

Figure 2. Laser scanning micrograph of lactococci treated with bovine milk immunoglobulins: WWA (a,b), E72 (c,d), SG1 (e,), M30 (g,h). a,c,e, and g = phase contrast; b,d,f, and h = fluorescent images stained with fluorescein conjugated bovine anti IgG. Magnification 4000x.

cultures E72 and M30 showed the greatest affinity to bind IgG. This was shown by the bright spots on the fluorescent images (Figure 2 (d, h)). Phase contrast images of E72 and M30 showed clumping or chain formation typical of agglutinating cultures (Figure 2(c, g)). In contrast the agglutination resistant cultures WWA and SG1 bound less IgG than the agglutination sensitive cultures (Figure 2 (b, d)). Phase contrast showed WWA and SG1 in smaller chains with few clumps. As with the single strain cultures, a comparison of the fluorescent images shows that the agglutinating culture M27 binds more IgG antibodies than the agglutination resistant culture SG1 (Figure 2 (b, d) and Figure 2 (f, h)). This supports the ELISA data (Table 1) in which M27 binds twice as much IgG as the nonagglutinating SG1. The ELISA data showed that E72 bound more IgG than M27, this was supported by the results seen from the LSM in which the agglutinating culture E72 binds more IgG than M30 as evidenced by the bright fluorescence in Figure 2(d, h).

Scheuble et al. (1989) reported no correlation between bacterial chain length and agglutination. However, Scheuble et al. (1989) believed that strains with a tendency to form long chains and are sensitive to immunoglobulins will show an increase in defect occurrences under commercial production conditions. An example of this was (Table 2) when WWA, a nonagglutinating culture, binds similar amounts of IgM and IgG, in addition to more IgA than many of the minor agglutinating cultures M27, M30, 478, and 874. This was verified visually in Figure 2 (a, b) in which WWA, even after being exposed to colostrum antibodies still

consisted of small chains, whereas E72 and M30 appear as long chains or clumps as seen in Figure 2(c, d) and Figure 2(g, h). Due to the small chain size, the weight of the bacteria casein complex does not reach the critical mass (Milton et al., 1990) needed for the clumps to sediment to the bottom of the vat.

Over the years, various techniques have been used to determine agglutination during cheese production based on the physical characteristics or defects associated with agglutination. The main methods are: 1) sediment depth 2) microbial enumeration 3) top and bottom total solids 4) direct microscopic examination. This information allowed dairy processors to choose a corrective action to reduce agglutination. The main draw back to these four procedures is that they are performed in the vat while production occurs, thus corrections can only be made to future incubations with yield loss or batch failure occurring during the test. Due to the monetary consequences associated with these "in the vat techniques", three screening procedures have been developed to determine bacterial sensitivity to agglutination. However, these procedures require the operator to determine the endpoints and reaction intensity leaving subjective differences from operator to operator.

The traditional method for the quantitation of immunoglobulins has been radial immunodiffusion (Mancini et al., 1965). The drawbacks of this assay are low sensitivity, time consuming, and requires large volumes of anti-sera. In recent years, immunoassays have been replacing standard methods of food analysis due

to their sensitivity, specificity and ease of application. Immunoassays offer several advantages over conventional procedures. They can be very specific and extremely sensitive, a combination that can result in simpler and quicker protocol procedures (Rodrigues et al., 1990). The two most common immunoassays in the food area are the ELISA and radioimmunoassay (RIA). The ELISA is undoubtedly the most widely used form of immunoassay in food analysis due to its simplicity and suitability as a screening method. The ELISA is easy to perform, sensitive, specific and relatively inexpensive (Rodrigues et al. 1990). The main advantage that the ELISA holds over the RIA for the dairy industry is that the RIA does not require the use of radioactive isotopes which make the RIA assay difficult for every day industry application.

Emmons et al. (1963) observed that immunoglobulins are always present in bulk milk. In addition to this Zawistowski and Mackinnon (1993) showed that up to 89% of Canadian raw milk was contaminated with colostrum. Since antibodies are present in raw milk, a rapid method to screen cultures for agglutination sensitivity would be advantageous for the dairy industry. Thunell et al. (1984), suggested that the selection of starter bacteria from the nonagglutinating strains would be the most effective method to reduce agglutination. Using an ELISA to screen cultures offers several advantages over conventional procedures. Ability to screen large numbers of samples combined with simplicity, sensitivity, and specificity, make the ELISA an ideal vehicle for use in the dairy industry. In this study, a rapid, objective method to

screen large numbers of bacteria was developed. The ELISA technique's versatility lends itself to rapidly screening cultures without the expenses associated with an "in the vat technique." One main advantage to the ELISA procedure is the elimination of the subjective determinations made by the operator. The ELISA is an objective assay that uses optical densities to determine agglutination, and does not require extensive training as many procedures including the those developed by Emmons et al. (1965), Ellerman (1975), and Salih and Sandine (1984).

3.2 Effect of heat treatment on the stability of bovine milk immunoglobulins

3.2.1. Antibody stability to heat treatment as determined by the ELISA

The effects of heat treatment at 71, 75, 80 and 85°C on the capabilities of bovine IgM, IgG, and IgA to bind lactococci are presented in Figures 3-5. M30 and M27 are commercial cultures used for Cheddar cheese production and represent the extreme case of agglutination problems. The SG1 culture on the other hand was a Cottage cheese culture which typically does not agglutinate and was used as a negative control in this study. At a constant temperature, the percent binding of immunoglobulins decreased ($P<0.05$) as heating time increased for all immunoglobulins (Figures 3-5). This was most notable ($P<0.05$) with the highest temperature of 85°C in which percent binding to lactococci was reduced to near zero levels within the first 20 min of heat treatment. Furthermore, at a constant time, the percent binding of immunoglobulins to lactococci decreased ($P<0.05$) with increases in temperature (Figure 3-5). Percent binding of lactococci was highest ($P<0.05$) at the lowest temperature of 71°C and lowest ($P<0.05$) at the highest temperature of 85°C (Figure 3-5). IgM was still able to bind all three lactococci after 90 min of heating at 71°C (Figure 3). When heated to 75°C, IgM showed less affinity to bind the lactococci than when treated at 71°C. The same IgM immunoglobulin when heated at 80 or 85°C bound less lactococci than those treated to lower temperatures

as evidenced in Figure 3. The data in Table 3 shows that increasing the temperature from 71 to 75 or 80°C decreased ($P<0.05$) IgM binding to all three lactococci. The data (Table 3) shows that the binding ability of IgM to lactococci was reduced ($P<0.5$) dramatically at 80°C. Kanno et al. (1976) reported that heating for 10 min at 70°C destroyed almost all of the activity of IgM which they suggested was the primary agglutination factor. This research showed (Figure 3; Table 3) that bovine immunoglobulins are still capable of binding lactococci after heat treatments at higher temperatures and longer times than that recommended by Kanno et al. (1976).

Table 3. Heat stability of bovine IgM and its ability to bind to lactococci after heat treatment of 45 minutes.

	Starter Culture		
Heat Treatment (°C)	M30	M27	SG1
	Optical Density Mean[1]		
71	0.779 [a] (0.10)	0.838 [a] (0.08)	0.621 [a] (0.18)
75	0.419 [b] (0.14)	0.422 [b] (0.20)	0.450 [b] (0.17)
80	0.061 [c] (0.12)	0.102 [c] (0.13)	0.189 [c] (0.10)

[1] Optical Density Mean with standard deviation in parentheses.
Means with different superscript are statistically different ($P < 0.05$) for comparisons made within the same column. Results of 4 independent treatments.

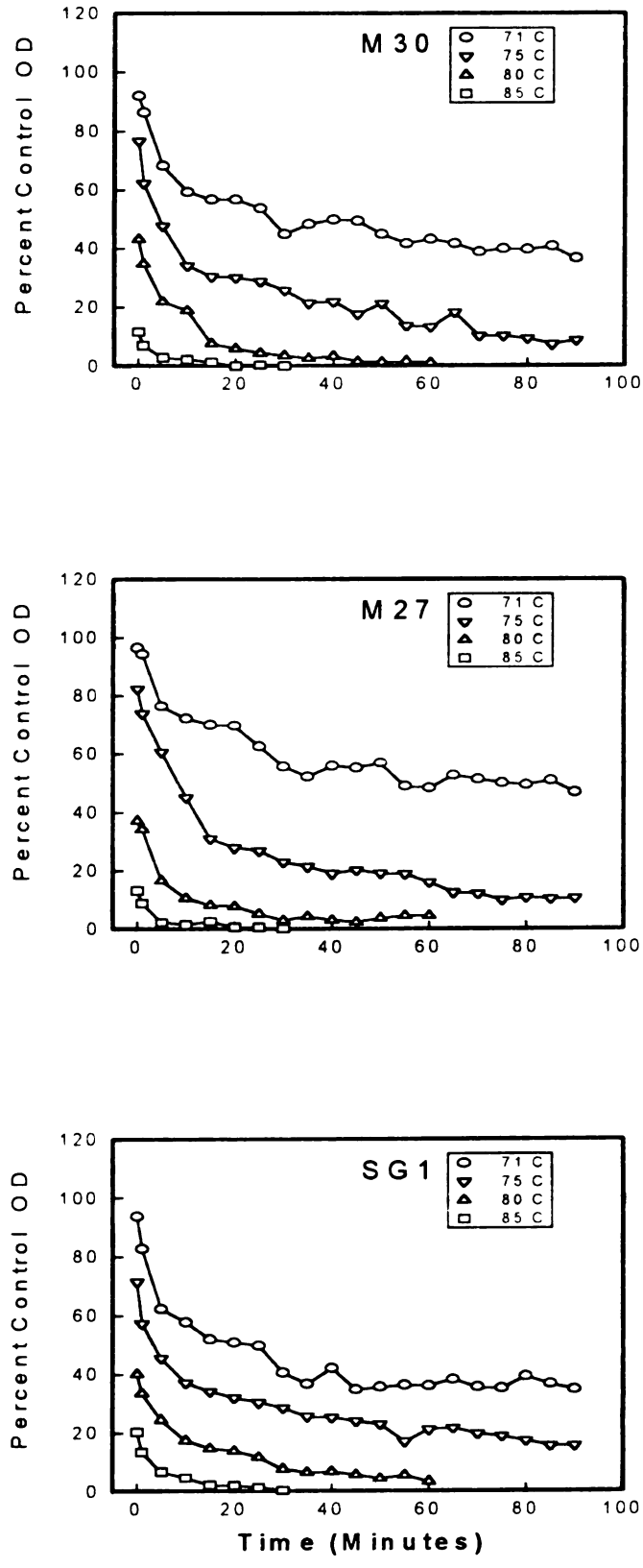

Figure 3. Heat stability of bovine IgM and its ability to bind lactococci as determined by an ELISA. Optical density in the unheated sample was 100%.

The bovine immunoglobulin IgG showed the greatest affinity to bind lactococci when heated to the lowest temperature of 71°C (Figure 4). When treated at 75°C, IgG affinity to bind lactococci decreased ($P<0.05$) as compared to IgG treated at 71°C (Figure 4; Table 4). The same antibodies when treated at 80 and 85°C, showed little affinity to bind lactococci as evidenced in Figure 4. Raising the temperature to 75 or 80 from 71°C decreased ($P<0.05$) IgG binding capabilities to lactococci (Table 4). My results are consistent with those of De Wit and Klarenbeek (1984) who reported that IgG was unfolded at 72°C in 0.7 M phosphate buffer, and also Fukumoto et al. (1994) who reported D values on bovine IgG of 329 min at 70°C, 38.8 min at 74°C, and 4.3 min at 80°C using radial immunodiffusion. Dominguez et al. (1997) determined that no loss in antigen binding of IgG occurred after 60 min at 65°C. My research revealed that the majority of the decreases in IgG binding occurred when the temperature was raised from 71°C to 75°C (Table 4; Figure 4). Dominguez et al. (1997) also reported that at 72°C it took only 1387 seconds for a 90% reduction in the binding activity of IgG and suggested that the ability of IgG to bind antigens after heat treatment has probably been overestimated in the literature. In my research, 71 or 75°C (Figure 4; Table 4) did not reduce immunoglobulin binding activity of IgG to the same extent as that reported by Dominguez et al. (1997). One difference between my research and the work of Dominguez et al. (1997) was the type of ELISA used where my research detected only those antibodies that bind lactococci, Dominguez et al. (1997) was interested

in all IgG immunoglobulins. Secondly, Dominguez et al. (1997) performed their heat studies on colostrum, whereas in this research, the heat treatment was performed on antibodies contained in a whey based media. Li-Chan et al. (1995) reported slightly higher D values for IgG than Dominguez et. al. (1997). Li-Chan et al. (1995) performed their research on serum IgG, which is composed of two subclasses of IgG (55% IgG_1 and 45% IgG_2). Whereas, bovine milk IgG is composed of 90% IgG_1 and 10% IgG_2. It is not known whether these subclasses show different denaturation behavior (Li-Chan et al., 1995). Li-Chan et al. (1995) concluded that a substantial portion of the antibody activity was retained in milk after commercial processing.

Table 4. Heat stability of bovine IgG and its ability to bind to lactococci after heat treatment of 45 minutes.

	Starter Culture		
Heat Treatment (°C)	M30	M27	SG1
	Optical Density Mean[1]		
71	1.159 [a] (0.30)	1.351 [a] (0.20)	1.235 [a] (0.36)
75	0.520 [b] (0.07)	0.503 [b] (0.09)	0.603 [b] (0.06)
80	0.167 [c] (0.05)	0.128 [c] (0.03)	0.195 [c] (0.10)

[1] Optical Density Mean with standard deviation in parentheses.
Means with different superscript are statistically different ($P < 0.05$) for comparisons made within the same column. Results of 4 independent treatments.

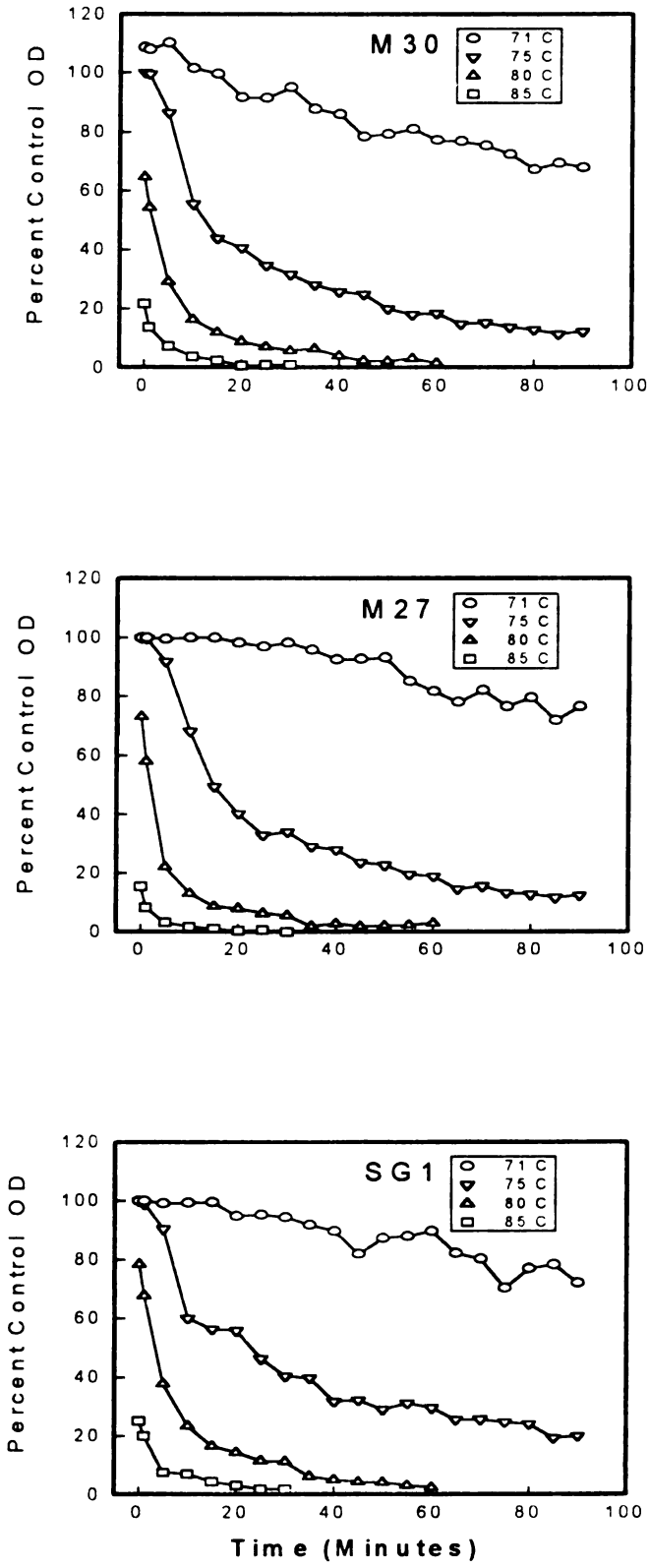

Figure 4. Heat stability of bovine IgG and its ability to bind lactococci as determined by an ELISA. Optical density in the unheated sample was 100%.

Table 5. Heat stability of bovine IgA and its ability to bind to lactococci after heat treatment of 45 minutes.

Heat Treatment (°C)	Starter Culture		
	M30	M27	SG1
	Optical Density Mean[1]		
71	0.072 [a] (0.01)	0.065 [a] (0.01)	0.072 [a] (0.01)
75	0.055 [a] (0.01)	0.049 [a] (0.01)	0.053 [a] (0.01)
80	0.022 [b] (0.03)	0.016 [a] (0.03)	0.034 [a] (0.01)

[1] Optical Density Mean with standard deviation in parentheses.
Means with different superscript are statistically different ($P < 0.05$) for comparisons made within the same column. Results of 4 independent treatments.

Of the bovine immunoglobulins IgA showed the least affinity to bind lactococci after thermal treatment as shown by the low optical densities at 71°C (Table 5). IgA showed the greatest affinity to bind lactococci at the lower temperatures of 71, and 75°C (Figure 5). When IgA was heated to temperatures greater than 80°C, the binding activity of IgA decreased ($P>0.05$) (Figure 5; Table 5). The data (Table 5) showed no statistical differences in IgA binding when the temperature was raised for commercial cultures M27 and SG1. Raising the temperature to 80 from 71 or 75°C decreased ($P<0.05$) IgA antibody binding to M30 (Table 5).

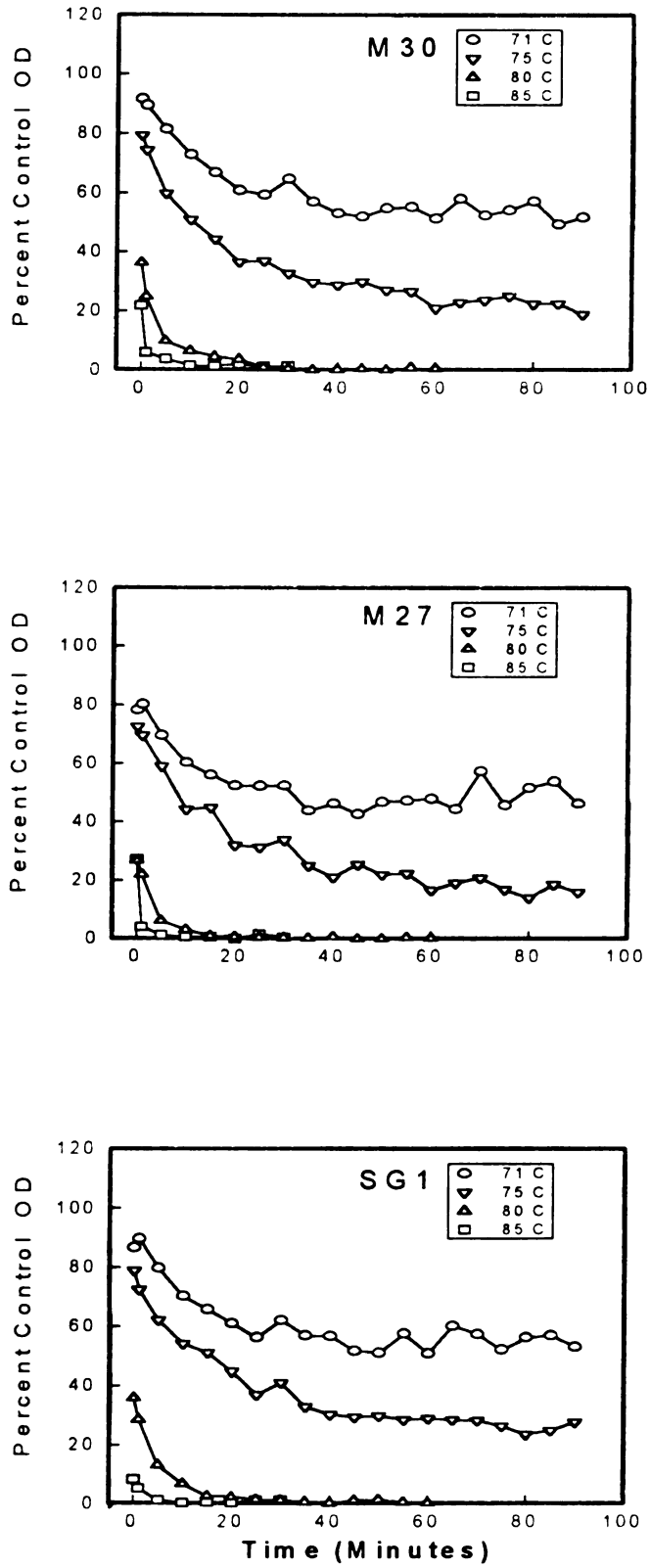

Figure 5. Heat stability of bovine IgA and its ability to bind lactococci as determined by an ELISA. Optical density in the unheated sample was 100%.

De Wit and Klarenbeek (1984) reported that reversible changes of protein structure mostly occur with temperatures up to 60°C. Lindstrom et al. (1994) concluded that thermal treatment of bovine immunoglobulins will cause the antibodies to unfold and aggregate. They reported that the unfolding of bovine antibodies due to heat at 80.9 ± 0.04°C is irreversible. This was consistent with Ruegg et al. (1977) who determined that the thermal unfolding of immunoglobulins was at 81°C in simulated milk ultrafiltrate. De Wit et al. (1983) reported that the immunoglobulin fraction denatured at 79°C at a 10% level in water. This was supported by the Figures 3-5 in which after heat treatment at temperatures greater than 80°C, the antibodies began to lose their binding affinity.

Differences in the results between this research and the literature data might be attributed to differences in the heating rate. Previous studies performed to determine the denaturation of immunoglobulins used volumes from 25 µl to 2 ml. My research used a larger volume of 50 ml needed to encompass the wide range of samples. This larger volume led to longer heating and cooling times which could have led to the inactivation of immunoglobulins. Also De Wit and Klarenbeek (1984), Lindstrom et al. (1994), Ruegg et. al. (1977) and De Wit et al. (1983) used differential scanning calorimetry (DSC) to determine protein unfolding, whereas my research focused on the effect of heat played on the binding ability of immunoglobulins to lactococci.

3.2.2. Antibody stability to heat as determined by laser scanning microscopy

Hicks and Ibrahim (1992) reported that the formation of long chains or clumps of bacteria are associated with bacterial agglutination. Whereas, smaller chain length and no clumping is associated with cultures that do not agglutinate. Some of the cells appears to be brighter than the rest (Figure 6f). This was explained by the fact that this lactococci is a mixed strain culture. The brighter fluorescence is due to the presence of an agglutination sensitive strain contained within an agglutination resistant culture. Autoclair and Vassel (1963) reported that mixed and multi strain cultures, although characterized as agglutination resistant may, contain agglutination sensitive strains which may become dominant upon extensive subculturing. Furthermore, although no phage problems were experienced in this research, the existence of phage could compound the agglutination problem if the agglutination resistant culture was destroyed, leaving the agglutination sensitive culture to complete the fermentation.

Microscopic analysis of the cultures with LSM using immunofluorescence to detect IgG, IgM, and IgA confirmed the ELISA results (Figures 6-8). Unheated immunoglobulins showed the greatest affinity to bind lactococci. This was shown by the heavy shining spots on the fluorescent images (Figures 6b, 7b, and 8b). The phase contrast images showed some clumping or chain formation of cells typical of agglutinating cultures (Figures 6a, 7a, and 8a). When immunoglobulins were heated at 71°C for 45 min the lactococci showed similar clumping and chain

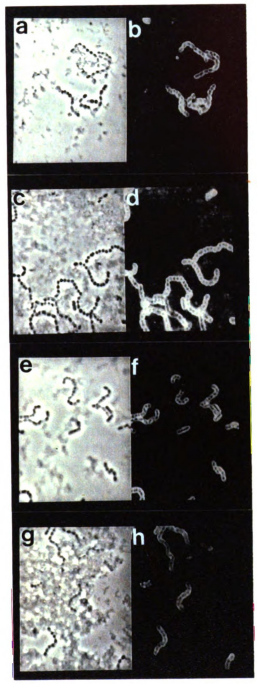

Figure 6. Laser scanning micrograph of lactococci with heat treated IgG: unheated control (a,b), heated at 71°C for 45 min. (c,d), heated at 75°C for 45 min. (e,f), and heated at 80°C for 45 min. (g,h). a,c,e, and g = phase contrast; b,d,f, and h = fluorescence, stained with fluorescein conjugated bovine anti IgG. Magnification 4000x.

length (Figures 6c, 7c, and 8c) as the untreated control (Figures 6a, 7a, and 8a). The affinity of IgM and IgA to bind lactococci decreased (Figures 7d and 8d) compared to the unheated control. Whereas, the affinity for IgG (Figure 6d) remained similar to the control as seen by the level of brightness (Figure 6b). When the immunoglobulins were heated at 75°C, the lactococci showed affinity to bind IgG, IgM, and IgA but to a lesser extent (Figure 6f, 7f, and 8f) than the unheated control and those heated at 71°C. Phase contrast showed smaller clumps and chain formation in comparison to unheated control. The immunoglobulins treated at 80°C showed very little or no binding to lactococci evidenced by very little or no binding of flourescein conjugated anti immunoglobulins. This is represented by the light to faint spots on the fluorescence images (Figures 6h, 7h, and 8h). Phase contrast showed lactococci forming short chains with little or no clumping.

Fukumoto et al. (1994) who reported that IgG was more heat stable than IgA due to the composition of bovine milk or the structure of IgG. My research supports the work of Fukumoto et al. (1994) as the binding abilities IgA was reduced when heat treated (Figures 8 (d, f, h)) as shown by little to no fluorescence. IgG was still capable of binding to lactococci after heat treatment at 80°C (Figure 6h) This correlates with the ELISA data where heat treated IgA retained little binding capabilities (Figure 8; Table 5). My research was consistent with that of Lindstrom et al. (1994), Ruegg et al. (1977), De Wit et al. (1983), in that the ELISA and LSM data show that the immunoglobulin affinity decreased quickly when heat treated to

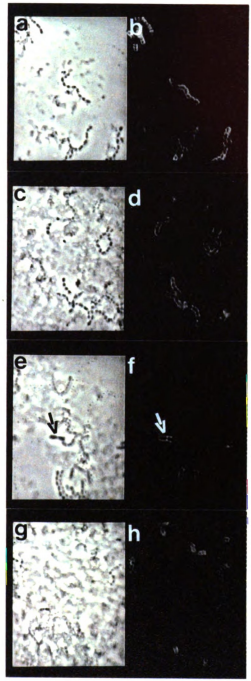

Figure 7. Laser scanning micrograph of lactococci with heat treated IgM: unheated control (a,b), heated at 71°C for 45 min. (c,d), heated at 75°C for 45 min. (e,f), and heated at 80°C for 45 min. (g,h). a,c,e, and g = phase contrast; b,d,f, and h = fluorescence, stained with fluorescein conjugated bovine anti IgM. Magnification 4000x.

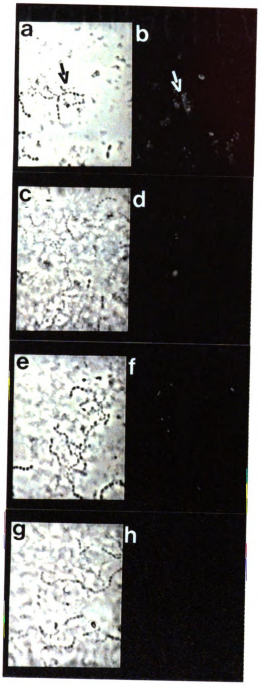

Figure 8. Laser scanning micrograph of lactococci with heat treated IgA: unheated control (a,b), heated at 71°C for 45 min. (c,d), heated at 75°C for 45 min. (e,f), and heated at 80°C for 45 min. (g,h). a,c,e, and g = phase contrast; b,d,f, and h = fluorescence, stained with fluorescein conjugated bovine anti IgA. Magnification 4000x.

temperatures above 80°C (Figures 3-5; 6(g, h), 7(g, h), and 8(g, h)).

3.2.3. Verification in skim milk

The rate of top and bottom pH decrease in pasteurized skim milk inoculated with lactococci grown in media heated at 71, 75, and 80°C is shown in Figure 9. The SG1 and M27 cultures had faster acid development and a smaller pH differential (Table 6) than M30 (Figure 9). Skim milk inoculated with either SG1 and M27 had a similar rate of acid production at the top and bottom of the graduated cylinder for heat treatments at 71, 75, and 80°C (Figure 9). Table 6 shows the pH differentials between top and bottom for skim milk at the same heat treatment. Milton et al. (1990) used a pH differential greater than 0.2 as an indicator of culture agglutination, whereas Hick and Ibrahim (1992) used 0.09 units to indicate agglutination. In this study a pH differential of 0.1 or greater was selected which was also used previously by Ibrahim (1995).

Neither SG1 or M27 showed signs of agglutination throughout the 5 hours of incubation when grown in media heated at 71, 75, and 80°C (Table 6). Visual inspection of the graduated cylinders at the end of the 5 h incubation showed no sediment in all heat treatments of M27 and SG1. Milton et al. (1990) reported that agglutination of lactococci can occur within the first 15 min of a cheese fermentation. These effects are also seen in the work reported here (Table 6) where after 2 h media heated at 71 and 75°C have surpassed the 0.1 pH differential set to

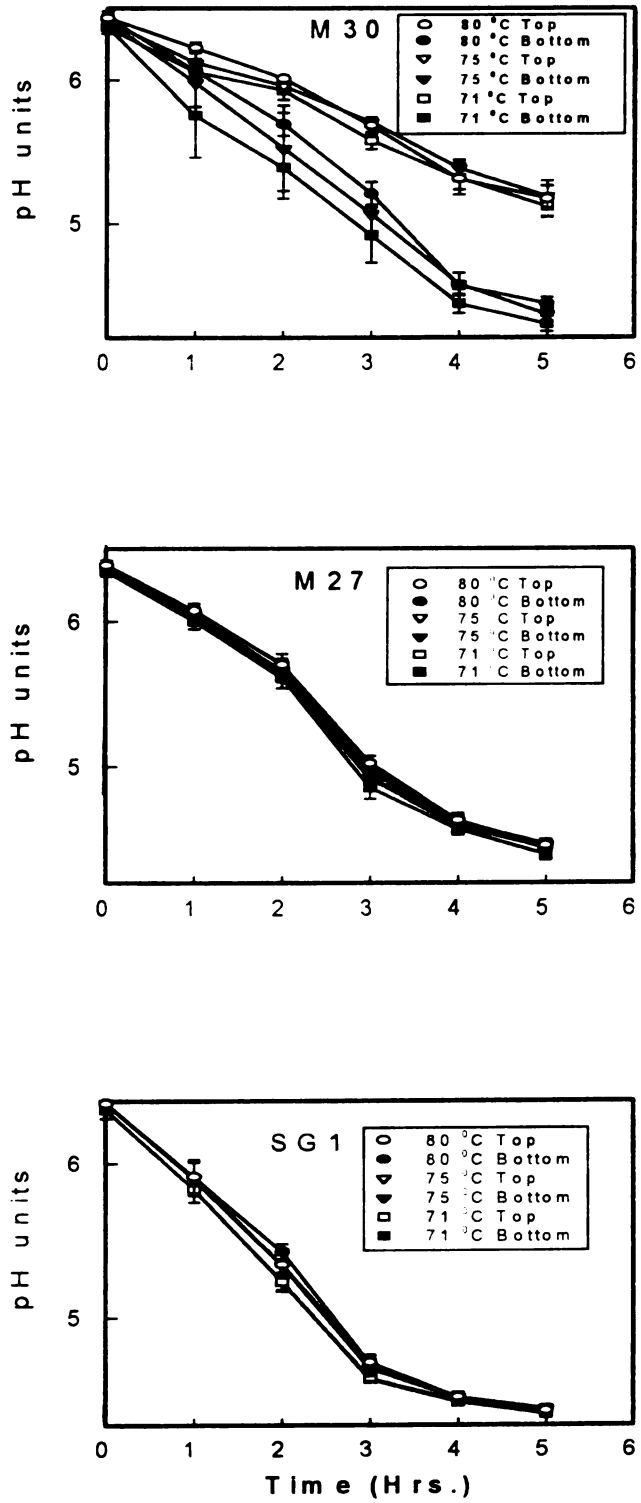

Figure 9. The effect of heat treatment on culture performance measured by pH differentials. Top and bottom pH decrease in 1000 ml cylinders of skim milk inoculated with lactococci grown in media heat treated at various temperatures.

indicate agglutination, whereas agglutination was not observed in the media heated to 80°C (Table 6). The agglutination prone M30 culture grown in the media treated to the higher temperature of 80°C shows less pH differential between top and bottom for the first 2 h of the incubation. However it was shown (Table 6) that M30 will agglutinate even at the high media pasteurization temperature of 80°C as seen in hours 3, 4, and 5 (Table 6). After 5 h a pH differential of 0.74, 0.74 and 0.61 was observed when M30 was grown in media treated to 71, 75 and 80°C, respectively. Visual inspection of the graduated cylinders incubated with M30 at the end of the 5 h of incubation had sediment on the bottom of the cylinder. The data suggest that heat treatment might prevent agglutination in the early stages of a fermentation and may provide a method to limit agglutination for bacteria that experience light to moderate agglutination. But if a culture is prone to agglutination, then it will do so because of immunoglobulins naturally present in the milk which have not received any additional heat treatment. An example of how heat may provide added protection against agglutination can be seen with the M27 culture which was characterized as an agglutination sensitive but shows little to no visible agglutination (Figure 9; Table 6). The data in Table 6 show that at the end of the 5 h incubation the samples treated at lower temperatures had greater agglutination characteristics than the those treated at 80°C. The trends observed in Table 6 were not statistically significant most likely due to the variability in pH between replications.

Table 6. The pH differentials[a] in 1000 ml cylinders of skim milk fermented with lactococci grown in media heated at various temperatures.

		Incubation Time (h)					
Heat Treatment (°C)	Culture	0	1	2	3	4	5
71	SG1	0.00	0.00	0.00	0.01	0.01	0.03
75	SG1	0.00	0.01	0.01	0.01	0.01	0.01
80	SG1	0.00	0.00	0.00	0.00	0.00	0.01
71	M27	0.00	0.01	0.02	0.05	0.04	0.05
75	M27	0.00	0.01	0.02	0.05	0.05	0.04
80	M27	0.00	0.01	0.01	0.02	0.02	0.02
71	M30	0.00	0.05	0.29	0.50	0.80	0.74
75	M30	0.00	0.05	0.17	0.49	0.70	0.74
80	M30	0.00	0.03	0.05	0.36	0.60	0.61

[a]pH differentials = top pH - bottom pH. Results are a mean of 4 independent treatments. Data within treatments were not statistically different.

M30, M27, and SG1 are multiple strain commercial cultures made from a defined number strains of lactococci. Although the bacteria were propagated from the same can of starter culture it is believed that during the individual replication that one strain or another became the dominant strain. Since each strain has a different affinity for agglutination, it is believed that this caused the larger variation in the data evidence by the large standard deviations.

The data reported here suggested that heating media and antibodies to 71°C, although sufficient for pasteurization, was not entirely effective to reduce the immunogenicity of the milk antibodies that cause agglutination (Figures 3-5). This study showed that bovine immunoglobulins are still capable of binding lactococci after heat treatments at higher temperatures and longer times than that recommended by Emmons et al. (1966); Kanno et al. (1976) and Dominguez et al. (1997). An increase in heat treatment to 75°C or 80°C may prevent agglutination in the bulk media.

3.3. Enzyme Treatment of immunoglobulins to reduce agglutination.

3.3.1. Antibody stability to enzyme treatment as determined by the ELISA

The effect of papain treatment on the capabilities of bovine IgM, IgG and IgA to bind lactococci are presented in Figure 10. The binding affinity of IgM was highest ($P<0.05$) at the lowest concentration 27.1 U/nl of papain and lowest ($P<0.05$) at the higher concentrations of 108 U/nl and 135.6 U/nl (Figure 10). This trend was also similar for IgG, however IgA showed the largest ($P<0.05$) binding at 0 U/nl and than less binding as papain concentration increased. The binding ability of IgM and IgG increased ($P<0.05$) (Table 10; Figure 10) after treatment at the lowest papain concentration of 27.1 U/nl (Figure 10). This was consistent with the theory that brief exposure of a protein to papain will cleave the most susceptible bonds, whereas prolonged exposure will cause the protein to become totally digested (Kuby, 1992). Brief exposure to papain will cleave immunoglobulins into monmers, as well as at the hinge region of the "Y" leaving 2(Fab) fragments and a tail section (Kuby, 1992). The monomers and Fab fragments with intact antigen receptor site were still capable of binding bacteria, and account for the increased binding of immunoglobulins (Figure 10). The decrease in the positional hindrances led to greater antibody binding (Figure 10; Table 7). However, as larger concentrations of papain were used more of the Fab fragments were digested leaving less antibodies

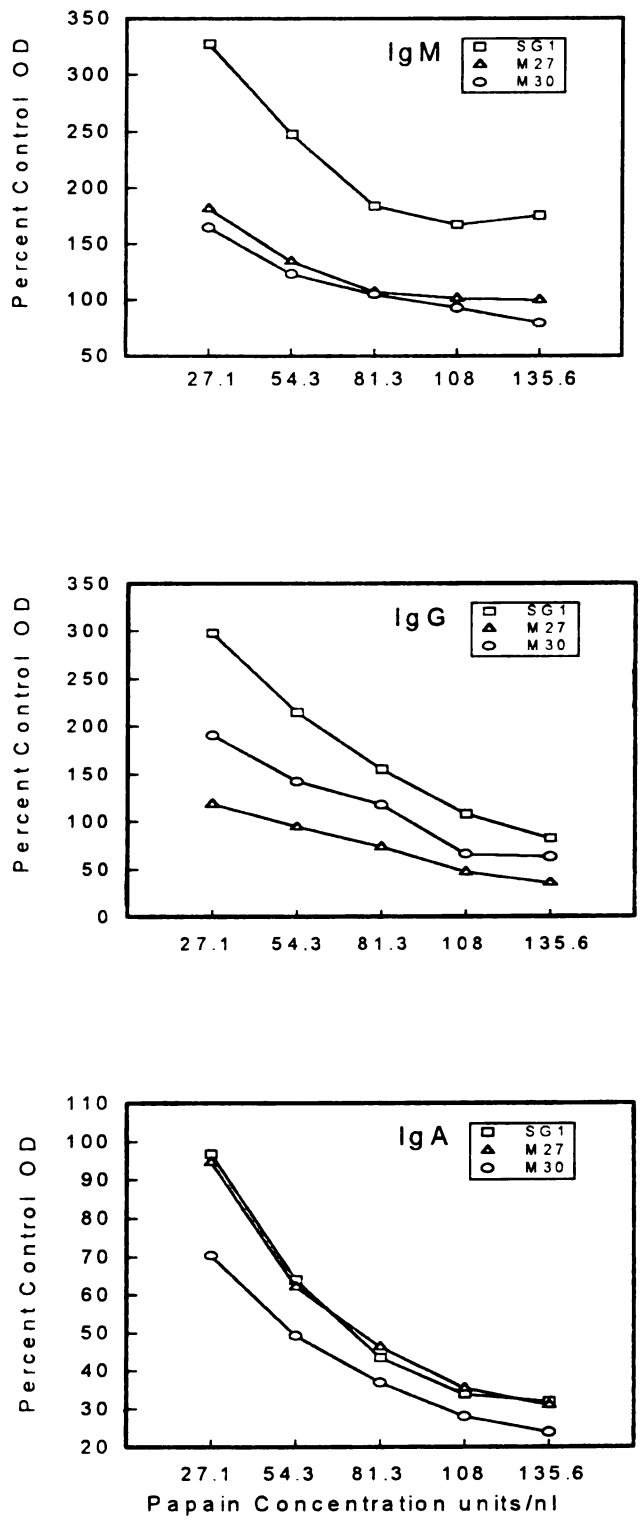

Figure 10. Effect of papain treatment for 2 h at 37°C on the stability of bovine IgM, IgG and IgA and their ability to bind lactococci as determined by an ELISA. Optical density in the nonenzyme treated sample was 100%.

Table 7. The stability of bovine IgM and its ability to bind lactococci after papain treatment at 37°C for 60 minutes.

Papain Concentration (units/nl)	Starter Culture		
	M30	M27	SG1
	Optical Density Mean[1]		
0	0.501[a] (0.12)	0.395[a] (0.11)	0.221[a] (0.14)
27.1	0.780[b] (0.08)	0.664[b] (0.11)	0.638[b] (0.16)
54.3	0.581[c] (0.07)	0.480[a] (0.08)	0.477[c] (0.12)
81.3	0.492[a] (0.07)	0.387[a] (0.05)	0.352[d] 0.08)
108.0	0.437[a] (0.06)	0.359[a] (0.05)	0.318[d] (0.07)
135.6	0.374[a] (0.04)	0.350[a] (0.06)	0.322[d] (0.08)

[1] Optical Density Mean with standard deviation in parentheses.
Means with different superscript are statistically different ($P < 0.05$) for comparisons made within the same column. Results of 4 independent treatments.

Table 8. The stability of bovine IgG and its ability to bind lactococci after papain treatment at 37°C for 60 minutes.

Papain Concentration (units/nl)	Starter Culture		
	M30	M27	SG1
	Optical Density Mean[1]		
0	0.503[a] (0.19)	0.642[a] (0.01)	0.296[a] (0.10)
27.1	0.833[b] (0.07)	0.761[b] (0.15)	0.818[b] (0.09)
54.3	0.673[c] (0.06)	0.605[a] (0.09)	0.604[c] (0.11)
81.3	0.519[a] (0.06)	0.474[c] (0.07)	0.436[d] (0.10)
108.0	0.308[a] (0.08)	0.304[d] (0.07)	0.314[a] (0.09)
135.6	0.270[a] (0.04)	0.228[d] (0.06)	0.236[d] (0.06)

[1] Optical Density Mean with standard deviation in parentheses.
Means with different superscript are statistically different ($P < 0.05$) for comparisons made within the same column. Results of 4 independent treatments.

Table 9. The stability of bovine IgA and its ability to bind lactococci after papain treatment at 37°C for 60 minutes.

	Starter Culture		
Papain Concentration (units/nl)	M30	M27	SG1
	Optical Density Mean[1]		
0	0.672[a] (0.14)	0.551[a] (0.13)	0.504[a] (0.06)
27.1	0.455[b] (0.06)	0.458[a] (0.05)	0.483[a] (0.03)
54.3	0.316[c] (0.06)	0.312[b] (0.04)	0.320[b] (0.03)
81.3	0.236[d] (0.03)	0.230[c] (0.03)	0.218[c] (0.02)
108.0	0.179[d] (0.03)	0.175[d] (0.02)	0.169[d] (0.02)
135.6	0.153[d] (0.01)	0.157[d] (0.01)	0.161[d] (0.02)

[1] Optical Density Mean with standard deviation in parentheses.
Means with different superscript are statistically different ($P < 0.05$) for comparisons made within the same column. Results of 4 independent treatments.

capable of binding lactococci. The data (Table 7; Figure 10) shows that the binding ability of IgM decreased ($P < 0.05$) as the papain concentration increased for the cultures M30, M27 and SG1.

The nonagglutinating culture SG1 bound less antibodies than M27 and M30 at the 0 U/nl concentration level. However after treatment at 27.1 U/nl, SG1 bound relatively similar amounts of immunoglobulins as the agglutinating cultures M30 and M27. Furthermore, SG1 bound a relatively greater percent when compared to the control than either M30 or M27. This may also be an indication that IgM is the major agglutinating factor (Kanno et al., 1976) because when the antibody is digested it eliminates the sterohindrances and thus SG1 binds relatively more IgM as a percent of the control than either of the agglutinating cultures M30 or M27. The results have shown a similar trend for IgG but a steeper decrease in antibody binding affinity when compared to IgM. As with IgM, SG1 bound the least amount of IgG antibodies at the 0 U/nl concentration level, but upon treatment at 27.1 U/nl, SG1 bound equal amounts of IgG as the agglutinating cultures M30 and M27. The data from the stability of bovine IgG and its ability to bind M30 (Table 8) did not pass the assumptions of a parametric test, therefore a nonparametric test based on ranks was performed leaving data which appears different, actually similar.

The effect of papain treatment on the binding ability of IgA to lactococci is shown in Table 9. The binding ability of IgA decreased ($P < 0.05$) as the papain

concentration increased for the cultures M30, M27 and SG1 (Table 9; Figure 10). The data in this table do not show the initial increase ($P<0.05$) in the binding ability of IgA at (27.1 U/nl) as IgM and IgG did. However as with IgM (Table 7) and IgG (Table 8) the binding capabilities of the antibody decreased when exposed to higher concentrations of papain (Table 9; Figure 10).

Ustunol and Hicks (1994) reported that papain digestion of antibodies decreased agglutination not only in the media but also in 1000 ml cylinders of skim milk. The initial increases in antibody binding shown in this research support the data reported by Ustunol and Hicks (1994) (Tables 7-9). Ustunol and Hicks (1994) reported that the hydrolyzed fragments specifically those less than molecular weight of 10,000 daltons bind the antigenic site on the cell surface in the same capacity as they did when they were part of the intact immunoglobulin. However, with the bridging mechanism removed the protein can no longer join bacteria to form chains or clumps. The blockers having effectively removed the antigenic binding site on the bacteria are then carried with the bacteria to the milk were they prevent milk antibodies from agglutinating the bacteria.

The effect of pepsin treatment on the binding capabilities of IgM, IgG and IgA to bind lactococci can be found in the Appendix (Tables 10-12; Figure 13). These results were inconclusive. Ustunol and Hicks (1994) also reported that proteolytic enzymes other than papain (such as trypsin, chymotrypsin, and pepsin) liberated peptides that do not inhibit agglutination. The data in Tables 10-12 had all

treatments similar for M30 and M27. This might be explained by the fact that the enzyme pepsin cleaves antibodies in the bond just above the interchain disulfide bonds. On an IgG molecule, pepsin produces one main fragment called $F(ab)_2$ fragment and many small fragments that compose the F_C. The $F(ab)_2$ fragment consists of the two Fab fragments joined by an interchain disulfide bond which can precipitate antigens (Kuby, 1992). This was shown in Figure 13 in which as the amount of pepsin increases, the percent binding of IgM and IgG remains statistically similar for M30, M27 and actually increases for the culture SG1.

3.3.2. Antibody stability to enzyme treatment as determined by Laser scanning microscopy

Microscopic analysis of lactococci with LSM using immunofluoresence to detect IgG confirmed the ELISA results (Figure 10; Table 7-9). Phase contrast images showed some clumping or chain formation typical of agglutinating cultures when treated with papain concentrations of 27.1 U/nl (Figure 11 (a, e)). The heavy binding of immunglobulin was evidenced by the large clumps of bacteria and the heavy fluorescence around the bacteria and clumps (Figure 11 (b, f)). When the immunoglobulins were treated at a concentration of 135.6 U/nl the phase contrast images of the lactococci showed less clumping and chain binding (Figure 11(c, g)). The fluorescent images at the higher papain concentration contain heavy spots of fluorescence for the agglutination resistant culture SG1 (Figure 11d).

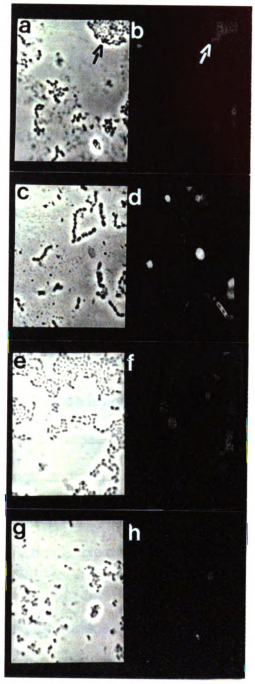

Figure 11. Laser scanning micrograph of commercial culture SG1 grown in Insure media after papain treatment at: 27.1 U/nl (a,b), 135.6 U/nl (c,d), culture M30 grown in Insure media after papain treatment at 27.1 U/nl (e,f), 135.6 U/nl (g,h). a,c,e, and g = phase contrast; b,d,f, and h = fluorescent images stained with fluorescein conjugated bovine anti IgG. Magnification 4000x.

Whereas the agglutination sensitive culture M30 appears as faint spots on the fluorescent images indicating little immunoglobulins are still present (Figure 11h). LSM results for the pepsin treatment are provided in the Appendix. As with the ELISA data there were no identifiable reductions in agglutination for either the phase contrast images (Figure 15 (a, c) and 15 (g, h)) or the fluorescent images (Figure 15 (b, d) and 15 (f, h)) after pepsin treatment of immunoglobulins.

In the LSM photomicrographs for papain (Figure 11), the transmitted image shows a decrease in agglutination between the 27.1 U/nl treatment and the higher papain concentration 135.6 U/nl treatment. At the higher papain concentrations (Figure 11 (c, g)) the decrease is characterized by smaller chains or clumps of bacteria. This supports the research of Ustunol and Hicks (1994) who saw a decrease in chain length and clumping of bacteria when grown in media treated with papain. When immunoglobulins were treated at the higher papain treatment of 135.6 U/nl their was a noticeable decrease in fluorescence for the agglutinating culture M30 (Figure 11(f, h)) . Less fluorescence means that fewer antibodies are still capable of binding the surface cell receptors of the bacteria resulting in less agglutination. This supports the theory that prolonged exposure of antibodies to papain will digest the entire antibody. These pictures (Figures 11(f, h)) support that theory.

3.3.3. Verification in skim milk

From the results of the ELISA and LSM, papain concentrations 27.1 U/nl, 81.3 U/nl and 135.6 U/nl were chosen for reduction of agglutination in a milk study. Unfortunately the enzyme was not denatured by the pasteurization step and quickly coagulated the milk leaving the milk study results inconclusive. Ustunol and Hicks, (1994) used diafiltration to isolate antibody fragments with a molecular weight less than 10,000 daltons. Commercial cultures OS, M30, and M37 when grown in media reconstituted with these hydrolyzed fragments showed reductions in agglutination of 40, 55 and 72% respectively. Diafiltration does represent a method to collect the hydrolyzed proteins from papain digestion while separating out the enzyme (Ustunol and Hicks, 1994). However attempts to use diafiltration to remove the enzyme in this milk study was unsuccessful due to equipment limitations.

Conclusions

Screening Study

The ELISA can be used to routinely screen large numbers of samples both quickly and objectively. The ELISA provided a rapid method to determine antibody binding capabilities, and thus, the agglutination behavior of lactococci. The ELISA results suggested that IgA plays a minor role in agglutination, whereas the role of IgG in agglutination may have been understated in the literature to date.

Heat Study

Heat treatment represented a method to denature antibodies as seen by both the ELISA and laser scanning microscopy. At a constant temperature, the percent binding of immunoglobulins to lactococci decreased as time of treatment increased. Additionally, decreases in the percent binding of immunoglobulins to lactococci was observed with increased temperature at a constant time. The data reported here suggested that heating media and antibodies to 71° C, although sufficient for pasteurization, was not effective to reduce the immunogenicity of the milk antibodies that cause agglutination. An increase in heat treatment to 75°C or 80°C caused decreases ($P<0.05$) in IgM, IgG and IgA binding to lactococci. Thus, increasing heat treatment could help reduce agglutination in bulk milk.

Enzyme Study

Papain treatment of immunoglobulins represented a method to denature antibodies as seen by the ELISA and laser scanning microscopy. However, the peptides released from the denaturation of antibodies with pepsin did not prevent agglutination. Upon partial digestion with papain all three antibodies were still able to bind lactococci. Additionally, initial increases in binding to M30, M27 and SG1 were observed for IgM and IgG but not IgA which showed decreases in antibody binding.

Recommendations

Continued investigation of the heat and enzyme studies with single strain cultures, as well as the evaluation of other enzymes such as chymopapain, trypsin, and chymotrypsin for their effect on antibody stability would be a benefit to the literature. The use of laser scanning microscopy should be investigated with splitscreen imaging and multiple antibodies to illustrate the relationship of agglutination between antibodies. Further investigation into the use of ultra violet light to kill bacteria, or chemicals such as formalin reduce the time needed for ELISA coating would be beneficial in decreasing assay preparation time.

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APPENDIX

Appendix

Pepsin ELISA and laser scanning microscopy data

Table 10. The stability of bovine IgM and its ability to bind lactococci after pepsin treatment at 37°C for 60 minutes.

	Starter Culture		
Pepsin Concentration (units/nl)	M30	M27	SG1
	Optical Density Mean[1]		
0	0.452[a] (0.11)	0.459[a] (0.05)	0.255[a] (0.11)
375	0.394[a] (0.15)	0.450[a] (0.09)	0.218[a] (0.08)
750	0.343[a] (0.12)	0.407[a] (0.08)	0.187[ab] (0.04)
1,100	0.333[a] (0.09)	0.427[a] (0.08)	0.218[a] (0.08)
1,500	0.395[a] (0.08)	0.432[a] (0.10)	0.280[ac] (0.08)
1,880	0.380[a] (0.13)	0.444[a] (0.10)	0.286[ac] (0.07)

[1] Optical Density Mean with standard deviation in parentheses.
Means with different superscript are statistically different ($P < 0.05$) for comparisons made within the same column. Results of 4 independent treatments.

Table 11. The stability of bovine IgG and its ability to bind lactococci after pepsin treatment at 37°C for 60 minutes.

Pepsin Concentration (units/nl)	Starter Culture		
	M30	M27	SG1
	Optical Density Mean[1]		
0	0.640[a] (0.10)	0.699[a] (0.07)	0.401[a] (0.17)
375	0.718[a] (.117)	0.689[a] (0.07)	0.478[a] (0.17)
750	0.651[a] (0.07)	0.739[a] (0.02)	0.454[a] (0.10)
1,100	0.741[a] (0.04)	0.751[a] (0.06)	0.607[ab] (0.13)
1,500	0.794[a] (0.05)	0.816[a] (0.04)	0.554[ab] (0.17)
1,880	0.784[a] (0.05)	0.688[a] (0.22)	0.747[b] (0.06)

[1] Optical Density Mean with standard deviation in parentheses.
Means with different superscript are statistically different ($P < 0.05$) for comparisons made within the same column. Results of 4 independent treatments.

Table 12. The stability of bovine IgA and its ability to bind lactococci after pepsin treatment at 37°C for 60 minutes.

Pepsin Concentration (units/nl)	Starter Culture		
	M30	M27	SG1
	Optical Density Mean[1]		
0	0.490[a] (0.25)	0.426[a] (0.22)	0.385[a] (0.24)
375	0.278[a] (0.12)	0.228[a] (0.09)	0.169[a] (0.11)
750	0.258[a] (0.09)	0.184[a] (0.06)	0.163[a] (0.09)
1,100	0.255[a] (0.08)	0.179[a] (0.47)	0.167[a] (0.08)
1,500	0.248[a] (0.08)	0.181[a] (0.49)	0.170[a] (0.06)
1,880	0.250[a] (0.08)	0.177[a] (0.05)	0.159[a] (0.06)

[1] Optical Density Mean with standard deviation in parentheses.
Means with different superscript are statistically different ($P < 0.05$) for comparisons made within the same column. Results of 4 independent treatments.

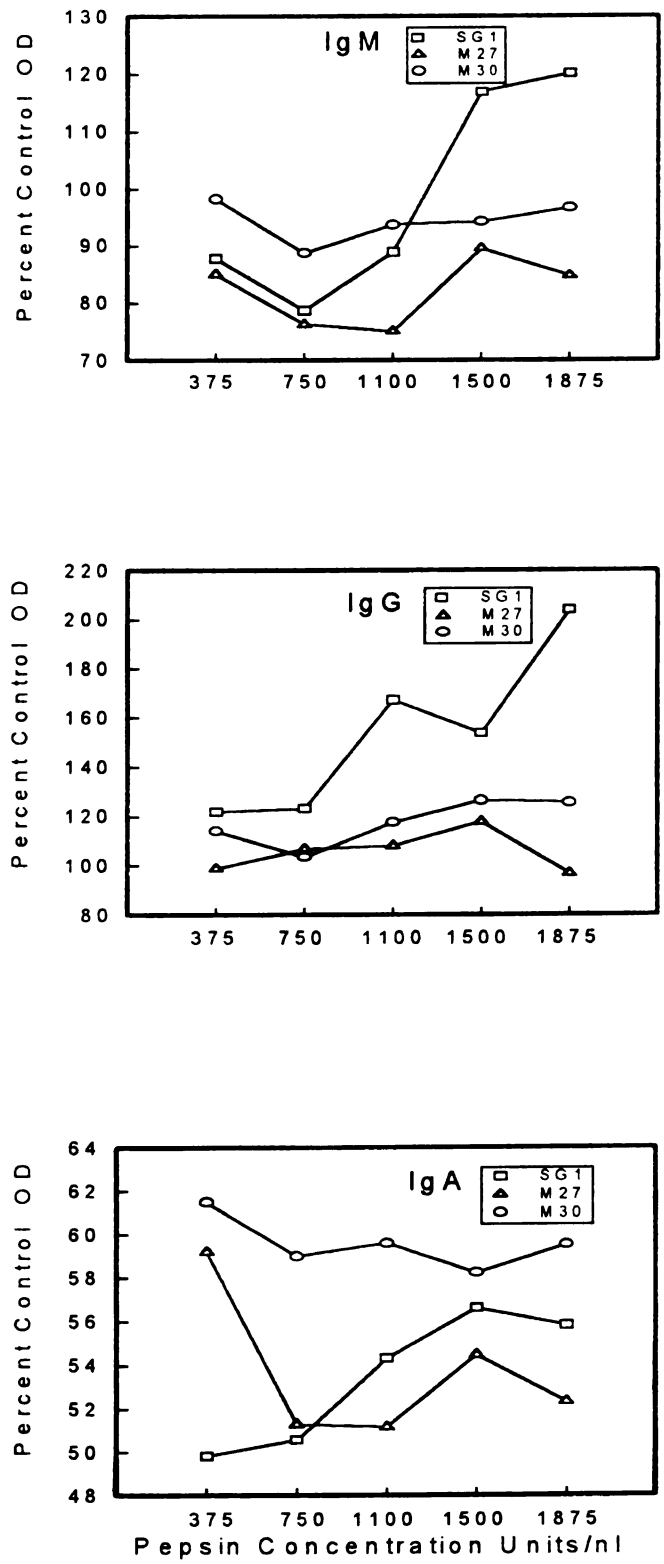

Figure 12. Effect of pepsin treatment for 2 h at 37°C on the stability of bovine IgM, IgG and IgA and their ability to bind lactococci as determined by an ELISA. Optical density in the nonenzyme treated sample was 100%.

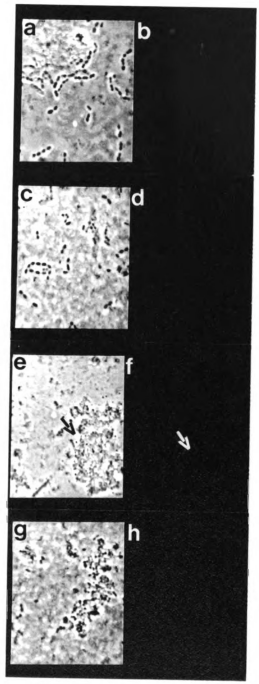

Figure 13. Laser scanning micrograph of commercial culture SG1 grown in Insure media after pepsin treatment at: 375 U/nl (a,b), 1,880 U/nl (c,d), and culture M30 grown in Insure media after papain treatment at 375 U/nl (e,f), 1,880 U/nl (g,h). a,c,e, and g = phase contrast; b,d,f, and h = fluorescent images stained with fluorescein conjugated bovine anti IgG. Magnification 4000x.