STUDIES ON HUMAN MILK: EFFECT OF HEAT TREATMENT AND
ULTRASONICATION, AND SEPARATION OF E. COLI O111: B4 LPS SPECIFIC IgA.

BY

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ABSTRACT

Human milk banks have been established in children's hospitals around the world to provide a steady supply of expressed donor human milk (EHM) to premature or sick infants. There are, however, two major technical concerns regarding feeding of EHM to premature neonates: a) possibility of disease transmission and b) problem of fat separation during tube feeding the babies.

A method using combination of ultrasonic waves and heat was developed for simultaneous pasteurization and homogenization of human milk. By using a sonicator which produced 600 W/in² energy at output control setting of 10 with a 3/8th inch disrupter horn, 80 mL of human milk in a bottle submerged in a hot water bath (93°C) could be heated to 72°C in 90 sec. The process was capable of inactivating $10^7$ CFU/mL of *Staphylococcus aureus* while retaining 80±2% of immunoglobulin A activity. The processed milk was adequately homogenized based on recovery of fat seen after mechanical infusion of milk, used as a simulation of tube feeding in clinical practice. In addition, the size of fat globules in human milk was reduced after thermo-ultrasonication to < 2.5μm which could further explain the stable fat recovery observed upon infusion.

Microwave heating patterns of 60 mL of water were studied to evaluate feasibility for pasteurization of human milk. Differences of ~7°C between the side and center of the sample bottle were observed. Immersing the sample bottle in a water bath to prevent surface heating did not reduce nonuniformity in heating. Constant agitation of the sample during heating was the best way of ensuring homogeneous temperatures and also resulted in rapid heating.

A continuous flow, small scale, economical heat processing device was also studied for rapid and efficient pasteurization of human milk. Human milk inoculated
with *Escherichia coli* (10^6 CFU/mL) or *S. aureus* (10^7 CFU/mL) was heated at 71°C at flow rates of 5.9, 12.3 and 18.9 mL/min, corresponding to holding times of 18.5, 9.0 and 5.75 sec. All conditions inactivated more than 10^6 CFU/mL of *S. aureus* and more than 10^6 CFU/mL of *E. coli* and also resulted in negative alkaline phosphatase activity indicating complete pasteurization. Pasteurization at 71°C for holding times mentioned above, resulted in retention of 63-83% of IgA, 58-79% IgG and 49-72% IgM which are comparable to batch pasteurization procedures used in human milk banks. A 30% residual activity of γ-glutamyl transpeptidase (GGTP) was found in bovine milk pasteurized at 71°C. Lower residual activity could therefore be used as an indicator of overpasteurization.

An affinity chromatography technique was developed for separation of enteropathogenic *E. coli* 0111:B4 LPS specific IgA from human milk. The affinity column was prepared by immobilizing LPS on porous chitosan beads using gluteraldehyde activation. The stability of immobilized LPS was confirmed upon repeated uses of the column with negligible leaching of the ligand. The column was able to bind 0.22 mg of LPS-specific IgA per mL. The affinity purified specific IgA accounted for 12.3% of total IgA and was found to cross react highly with *E. coli* 0128:B12, and *Klebsiella pneumoniae* using LPS-ELISA. The anti-LPS Ab separated might have applications for prevention or treatment of gram-negative infections in high risk individuals. The technique might be used for separation of LPS specific antibodies for treatment or diagnosis of gram-negative bacteremia.
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Chapter 1

Introduction

Human milk is desirable for all babies but there are some, both in hospital and at home, for whom special efforts should be made to supply human milk because of its unique nutritional and immunological properties (Schanler and Hurst, 1994; Ebrahim, 1995). These babies may be preterm, small or sick, those who are recovering in early infancy from surgery, those in whom there is a family history of atopy or who are otherwise at a risk of cow's milk intolerance and those who are unable, for short periods, to feed from their mothers (Department of Health and Social Security, 1981).

Providing a steady and safe supply of human milk for situations described above, however, is not a simple matter. There are issues regarding bacterial and viral contamination of donor human milk and the underlying possibility of disease transmission (Ruff, 1994; Lin et al., 1988; Lucas and Roberts, 1979). Heat treatment of human milk therefore has been highly recommended, especially for donor human milk (Arnold and Tully, 1992). The types of pasteurization equipment commercially available for human milk are very limited. Those available utilize only low temperature, long time procedures, need large volumes (4 L to 10 L of human milk) to operate economically and are also very expensive. In addition, there is no
commercially available equipment for pasteurization of individual milk samples at one time. There is also a paucity of literature regarding alternate forms of heat treatment, such as high temperature short time treatments (HTST), which have the potential for better retention of the nutrients and immune factors of milk (Morgan et al., 1986; Eitenmiller, 1991). In view of the above, some of the objectives of this thesis have been to study alternate forms of heat treatment that would lead to rapid pasteurization of human milk and also permit high retention of immune factors. Use of microwaves, ultrasonic waves and a continuous flow HTST pasteurization system have been studied (chapter 3, 4 and 5) to that effect.

For premature or ill infants who have to be fed nasogastrically, there is concern about the separation and loss of fat from human milk during continuous mechanical infusions (Greer et al., 1984; Stocks et al., 1985). Since lipids in human milk are specially suited for the need of infants (Hamosh, 1994), and also account for approximately 50% of the calories (Garza et al., 1987), loss of milk fat is highly undesirable. Since ultrasonication has been shown to prevent fat loss in human milk during tube feeding (Martinez et al., 1987), use of ultrasonic waves in combination with heat (thermo-ultrasonication), has been studied in this investigation for possible simultaneous pasteurization and homogenization of human milk (chapter 4).

Infants around the world have been known to be susceptible to infectious diseases owing to their immature immune system. Most often severe illnesses following gram-negative bacterial infections, such as bacteremia and necrotizing
enterocolitis occur in infants who are already immunocompromised (Miser et al., 1981; Bonadio et al., 1991). Passive immunization by oral administration of antibodies from human serum has been shown to be effective as a prophylactic measure against rotavirus and necrotizing enterocolitis infections (Eibl et al., 1988; Barnes et al., 1982). Administration of intravenous immunoglobulins (IVIG) enhanced survival in septic neonates of low birth weight (Sidiropoulos et al., 1986). Human milk has been known to confer protection in infants against infection due to its specific antibody activity (Glass et al., 1983; Cruz et al., 1988; Hayani et al., 1992). In this investigation, a technique was developed for separation of specific IgA antibody to the lipopolysaccharide (LPS) of enteropathogenic Escherichia coli using LPS immobilized affinity chromatography (chapter 6).
Chapter 2

Literature Review

This literature review will begin with a discussion of the clinical importance and uses of expressed human milk (EHM), following which some of the issues concerning feeding of EHM, such as the problem of bacterial contamination, heat treatment of EHM and its effect on immune factors of breast milk will be reviewed. The latter part of the review will focus more on the specific antibody activity of human milk, and there will also be some discussion on production and uses of anti-lipopolysaccharide antibodies.

2.1 Expressed human milk (EHM): Clinical applications

Banked expressed human milk has been advocated for treatment of numerous conditions in patients ranging in age from very premature neonates to young adults. Although, in some cases, evidence of the efficacy of human milk as a therapeutic agent appears anecdotal, there is nevertheless a considerable body of more substantial clinical evidence for its use in certain instances. Especially in recent years, new data have emerged to support the rationale for feeding
expressed human milk to hospitalized preterm and other high risk infants (Lucas and Cole, 1990; Lucas, 1993; Malloy and Graubad, 1994).

Perhaps the most common condition for which banked human milk is prescribed are those directly related to infant nutrition (Pitt, 1979; Pittard et al., 1985), such as allergy or intolerance to other formulae (Lucas, 1993) and failure to thrive (Maclean and Fink, 1980). It has also been advocated for the alimentation of newborns possessing various birth defects (Nayman et al., 1979), such as transient-neonatal tyrosinemia (Goldman et al., 1974) and acrodermatitis enteropathica (Branski, 1980). Patients recovering from surgical procedures for gastroschisis, intestinal obstruction with bowel fistulae, and renal insufficiency have also benefitted from banked milk (Asquith et al., 1987).

During the past 15 years, considerable evidence has accrued regarding the important role the immune system of human milk plays in protecting not only the full term baby, but also the premature infant who is more prone to infections and damage caused by inflammatory processes. Banked human milk has been successfully used as a prophylactic agent against necrotizing enterocolitis in recent years (Asquith et al., 1987; Lucas and Cole 1990; Lucas, 1993). In a randomized controlled trial, holder pasteurized expressed human milk conferred substantial protection against neonatal infection (Narayanan et al., 1984). In fact, banked breast milk has been prescribed for a wide range of infectious diseases such as chronic intractable diarrhea (Narayanan et al., 1982), gastroenteritis (Ste. Marie et al., 1974) and ulcerative colitis (Pittard et al., 1985), where it was claimed
to have produced dramatic results. Even supplemental feeding of expressed breast milk has been associated with a reduced frequency of infections in the low birth weight infants (Yu et al., 1981; Narayanan et al., 1980). One recent report on the failure of intravenous human immunoglobulin to prevent or ameliorate sepsis in premature infants (Magny et al., 1991) may encourage more widespread use of expressed human milk feedings in those populations.

Another possible therapeutic indication for the use of human milk has been in the treatment of diseases caused by enteric pathogens. Single donor, unpasteurized banked milk has been utilized in the treatment of infants with both serologic and secretory IgA deficiency, who were chronically infected with intestinal pathogens such as *Giardia lamblia*, rotavirus (Liebhaber, 1983; Gillin et al., 1985) and *Clostridium botulinum* (Narayanan et al., 1981). Since an enteromammary link in SlgA production and immunological memory of previously encountered antigens in the human secretory immune system has been strongly suggested (Nathavitharana et al., 1994; Nathavitharana et al., 1995) the above documented therapeutic impact of expressed breast milk becomes more understandable.

Allergy or intolerance to infant formulae is a common problem encountered in the feeding of premature infants. Immediate and even short term use of banked breast milk prevents the need for parenteral nutrition because enteral feeding is rapidly established (Balmer and Wharton, 1992). This could offer an important benefit when breast milk is used as the first enteral feed after neonatal bowel
Lucas (1993) reported better feeding tolerance, with faster establishment of full enteral feedings in expressed breast milk fed premature infants (20 days with expressed breast milk vs 48 days with preterm formula and 45 days with standard formula). Human milk/colostral antibodies provide the immature gut of the infant with immunity and perhaps protection, against sensitization through the binding of IgA antibody to foreign protein (Walker, 1980). Many of the growth factors present in human milk may also serve an important role in the treatment of allergic and immunologic disorders in the older child (Garza et al., 1987). Banked breast milk has been fed to patients with known or suspected allergy to bovine milk or commercial milk substitutes (Davies and Evans, 1978; Kwock et al., 1984).

The influence of diet on neurobehavioural development is a new area of interest and particular concern (Schanler and Hurst, 1994). Some recent studies show significantly higher intellectual development in children who had been fed human milk as a preterm baby compared to those who were never fed human milk or were receiving preterm or fullterm formulae (Lucas et al., 1992; Rogan and Gladen, 1993; Lucas et al., 1994). Further studies in these areas are needed to understand the mechanism related to these findings.

2.2 Issues concerning feeding of banked expressed human milk

2.2.1 The problem of bacterial contamination

The previous section amply shows the benefits of providing human milk to the high risk premature, sick infant, or infants who are allergic to cow's milk based
formulae. To provide this nourishment to infants, human milk banks have been established in the United States, Canada, Europe and elsewhere. Human milk however, is highly perishable and also susceptible to bacterial contamination which may occur during pumping milk from the breast, collection, transport or milk storage. Exposure to microbial contaminants may be related to organisms on mother’s skin or hands, breast-pump components, or milk containers (El-Mohandas et al., 1993b). It has been suggested that contamination may also occur in the hospital environment during the final stages of preparation and feeding of the milk to the infant (Costa, 1989).

The bacteriological profiles of fresh human milk samples may vary one from the other due to the donor’s hygiene, collecting and handling methods, and environmental conditions (Baum 1982; Goldblum et al., 1984; Lucas and Roberts, 1979). Gavin and Ostovar (1977) studied bacterial population in the milk samples of five mothers. *Staphylococcus. epidermidis* was the predominant organism isolated from 100% of the samples. Lucas and Roberts (1979) examined six pools of milk donated to a milk bank and found two to be contaminated with *E. coli* (10^7 and 10^5 CFU/ml), one with *S. aureus* (10^5 CFU/mL), and one with β-hemolytic streptococci (10^5 CFU/mL). Other non pathogenic bacteria isolated and identified included *Bacillus* spp., coagulase negative staphylococci, lactobacilli, *Pseudomonas acnes*, non fecal gram-negative bacilli and *Streptococcus faecalis*. Lin et al. (1988) compared bacteriological profiles of human milk samples collected under supervised conditions from individual donors to pooled human
milk samples obtained from a human milk bank. All individual samples were free of coliforms, and the average total aerobic count was $1.5 \times 10^3$ CFU/mL. In the pooled samples, four out of five samples contained coliforms with an average of $6.8 \times 10^5$ CFU/mL and the mean aerobic count of these samples was $2.9 \times 10^6$ CFU/mL which therefore indicated a higher contamination of pooled samples over individual samples. When comparison was based on the identity of each isolate, the pooled samples contained a more diverse microflora with a greater potential for pathogenic bacteria to be present. *S. epidermidis* was the microorganism that was isolated most frequently from either individual or pooled samples. Interestingly, *S. epidermidis* is emerging as the leading cause of nosocomial sepsis in intensive care nurseries (Anday and Talbot, 1985). Furthermore, El-Mohandas et al. (1993a) showed a significant association between *S. epidermidis* sepsis and: a) gastrointestinal colonization with the same organism and b) diagnosis of necrotising enterocolitis.

Overall, ranges and averages of the bacterial counts varied from one study to another and it could probably result from the method used to collect or handle the sample or the hygiene of the donor. Several studies have shown that proper hygiene of donors and more aseptic collection procedures will result in lower initial bacterial counts (Lucas and Roberts, 1979; Carroll et al., 1980; Lin et al., 1988). However, one study suggested no difference in the number of heavily contaminated (10,000 CFU/mL or more) milk cultures when a clean versus a sterile collection container was employed and when a manual versus a mechanical
collection technique was used (Pittard et al., 1991). Since most bacteria found in the milk are thought to originate from the skin, it has been suggested that discarding the initial few mLs of milk from the breast would result in lower bacterial count in the final sample (Carroll et al., 1980; Pittard et al., 1985). However, the study by Pittard et al. (1991) showed that the initial milk specimen expressed did not have a significantly increased amount of bacterial contamination compared with later specimens expressed from the same breast.

Although several researchers have indicated that pasteurization of human milk is unnecessary (Bjorksten et al., 1980; Hoey et al., 1980; Reynolds and Lewis-Jones, 1982), and some have given suggestions to obtain a less contaminated milk sample (Pittard et al., 1985; Lucas and Roberts, 1979; Carroll et al., 1980) the prospect of feeding pooled donor milk presents a risk too great to tolerate. Besides bacterial infection, there is also possibility of transmission of viral infections, such as human immunodeficiency virus (HIV), hepatitis B and C and cytomegalovirus (CMV) (Ruff, 1994).

Therefore drawing together the diversity and numbers of bacteria present in the pooled milk samples, occurrence of pathogens and the possibility of transmitting viral infections, the need for some form of heat treatment to ensure the safety of the infant becomes evident.

2.2.2 Heat treatment of human milk

There are two concerns revolving around heat treatment of donor milk. If milk is not heat treated, there is concern regarding disease transmission. If milk is heat
treated, there is concern with the loss of nutrients and immunologic factors. Recently, the Human Milk Banking Association of North America (HMBANA) in conjunction with the representatives of the Centers for Disease Control and Prevention (CDC), The Food and Drug Administration, and the Infectious Disease Committee of the American Academy of Pediatrics developed guidelines that ensure the safety of donor breast milk while retaining its nutritional and immunologic qualities (Arnold and Tully, 1992). These guidelines focus on donor screening for medical history, donor serum screening, donor education and very importantly, heat treatment of the milk. Although these guidelines are not rules and are not legally enforced, they do recommend pasteurization of all donor milk specimens.

In the milk banks that follow the HMBANA guidelines, heat treatment is carried out at either 56°C or 62.5°C (holder pasteurization). Milk is brought to the desired temperature, held for 30 min and then rapidly chilled in an ice slurry, and refrozen. To confirm the effect of heat treatment a sample of pasteurized milk is bacteriologically tested. No bacteriologic growth in heat-processed milk is acceptable (Arnold and Larson, 1993). In some milk banks around the world, automated batch heating human milk pasteurizers are used. In the B. C. Children's Hospital in Vancouver, the Colgate Axicare™ CM85 human milk pasteurizer is used where milk is heated at 63°C for 30 min followed by a rapid cooling cycle. The pasteurizer can process up to 10 litres of milk per cycle but is also extremely expensive. In Sorrento Maternity Hospital in Birmingham, milk is pasteurized at
56°C for 30 min in a custom built pasteurizer (similar to the Colgate Axicare™ pasteurizer) and rapidly cooled to 10°C (Balmer and Wharton, 1992).

Holder pasteurization (62.5°C for 30 min) has been shown to effectively inactivate HIV-1 and CMV, and to eliminate or significantly decrease titers of most other viruses and bacteria (Orloff et al., 1993). In another study, holder pasteurization for 30 min at both 56°C and 62.5°C was shown to completely inactivate >10^6 E. coli and S. aureus per mL of human milk (Eitenmiller, 1991). In fact, most previous studies on effects of processing on bacteria in human milk have examined low temperature (62.5°C) heat treatments for 30 min (Eyres et al., 1978; Lucas and Roberts 1979; Raptopoulou-Gigi et al., 1977), or 15 min (Baum, 1973; Jones et al., 1979).

Information on the applicability of high-temperature short-time (HTST) pasteurization procedures (72°C for 16 sec) to human milk is very scarce. More specifically, very little information is available on the effects of HTST processing on the pathogenic bacteria in human milk. In two studies, a continuous flow high-temperature short-time pasteurization system was used to determine the rates of thermal destruction of bacterial pathogens, E. coli, K. pneumoniae (Lin et al., 1987) and S. aureus in mature human milk (Morgan et al., 1988). The HTST pasteurizer used in both studies was designed and built specifically for small volumes (< 50 mL to any volume desired). Perhaps one reason HTST heat treatments have not been thoroughly investigated as a procedure for
pasteurization of human milk is the lack of a heating device for effective treatment of small volumes. More studies in this area are clearly needed.

Due to the destruction of immune components reported with the holder pasteurization procedure (Liebhaber et al., 1977; Williamson et al., 1978; Eyres et al., 1978; Wills et al., 1982) some interest has also been shown in using microwaves for processing of human milk (Sigman et al., 1989; Quan et al., 1992). Although one study did report microwaving to be as effective as holder pasteurization in killing bacteria present in human milk (Sigman et al., 1989), more emphasis was placed on preservation of immunoglobulins than on inactivation of bacteria.

Since the use of mother's milk for the sick and premature infants is being highly advocated for several reasons, and with the knowledge of the risks associated with feeding of human milk, it becomes important to thoroughly investigate different heat treatment procedures and their effect on inactivation of bacteria, viruses and host defense factors in human milk.

2.2.3 Anti-microbial activity of human milk: Effect of heat treatment

Research in the past two decades has helped to establish human breast milk as a potent immunocompetent agent containing a rich variety of products, each one of which has a role to play in the immunological protection of the infant (Ebrahim, 1995). Wherever studies have been carried out, the protective effect of breast milk has been amply proven, though naturally it is best demonstrated in populations
living in poor sanitary conditions (Howie et al., 1990; Victoria et al., 1989). Studying the loss or inactivation of the protective factors in human milk due to processing therefore becomes extremely critical. However, prior to reviewing the effect of various heat treatments on the immunological activity of human milk, it seems relevant and important to briefly review those immune factors.

2.2.3.1 Immune factors of human milk

The major components of the immune system of human milk are: (a) antimicrobial factors, such as secretory IgA, lactoferrin, lysozyme, oligosaccharides and lipids, (b) anti-inflammatory agents, such as prostaglandins, epidermal growth factor and secretory IgA and (c) immunomodulating agents such as interleukins (Goldman, 1993; Ebrahim, 1995).

In the present review, however, the focus will be on immunoglobulins of human milk, more specifically secretory IgA (slgA) since it is the predominant immunoglobulin present in human milk, comprising more than 90% of all immunoglobulins (Hanson et al., 1984; Blanc, 1981). In early colostrum, slgA may be present in concentrations of 17 mg/mL, decreasing in 4 day colostrum to 1 mg/mL (Amman and Stiehm, 1966; Goldman, 1993); in bovine colostrum it is almost nil. In contrast to high concentrations of slgA in breast milk, small amounts of IgG and IgM are also present. Reported values of IgG are 0.4 mg/mL in initial colostrum and 0.04 mg/mL in 4 day colostrum (Amman and Stiehm, 1966; Blanc, 1981). IgM is found in human milk in lower concentrations than slgA, with values
of 1.6 mg/mL in initial colostrum and 0.1 mg/mL in 4 day colostrum (Ammann and Stiehm, 1966).

sIgA contains most of the antibodies synthesized by maternal lymphocytes in response to infections, food and other antigens (Mata, 1986). The presence of sIgA in human milk has been therefore shown to reflect prior exposure of the mother to the immunogenic stimuli, and the adequacy of the enteromammary circuit of her immune system (Hanson et al., 1984; Nathavitharana et al., 1995). Although not regarded as bactericidal per se, sIgA may prevent microbial attachment to host epithelial cells, otherwise interfere with bacterial replication or be inhibitory or even bactericidal when acting in concert with unsaturated lactoferrin (Dolan et al., 1989). sIgA antibodies directed against bacteria (Glass et al., 1983), bacterial components (Ruiz-Palacios et al., 1990), protozoa (Miotti et al., 1985) and viruses (Okamoto and Ogra, 1989) have been detected in human milk. Most of the IgA in human milk (~80%) is present in the dimeric form. It consists of two monomers (160 kDa each), linked by disulphide bonds and an additional, cysteine-rich polypeptide termed J chain (16 kDa). It also consists of a heavily glycosylated protein called the secretory component (50-90 kDa), which is complexed with IgA during the secretion process and appears to protect IgA from degradation by proteolytic enzymes, which is beneficial to its protective role in the digestive tract (Kerr, 1990).

IgG carries out a variety of functions similar to those of IgA. It is present in much higher concentrations in human serum than in milk and is involved in
neutralization of toxins and viruses and binding and opsonization of bacteria (Kimball, 1986). IgG has the same configuration as the IgA monomer, consisting of two heavy and two light polypeptide chains linked by disulphide bonds. IgM on the other hand is made up of five basic subunits (ten heavy and ten light chains) and the presence of large amounts of the amino acid cystine provides for a circular configuration. IgM shows strong defense against gram-negative pathogens like *E. coli* and *Salmonella* (Nestle, 1975).

### 2.2.3.2 Effect of heat treatment on the immunoglobulins of human milk

Since human milk consists of such potent antimicrobial factors, as were discussed above and more, which can be destroyed by excessive heating, choosing the appropriate heating temperature and time for pasteurization of milk becomes very important. The temperatures studied for pasteurization of human milk range from 56°C to 100°C but mostly employ bulk heating (Evans et al., 1978; Liebhaber et al., 1977, Gibbs et al., 1977; Wills et al., 1982; Goldsmith et al., 1983; Eitenmiller, 1991). Literature values for the retention of immunoglobulins vary widely among investigators. Retention of IgA at 62.5° or 63°C for 30 min (lower temperature long time) fluctuated between 51% (Eitenmiller, 1991), 67% (Wills et al., 1982), 78% (Ford et al., 1977), 86% (Raptopoulou-Gigi et al., 1977) and 100% (Evans et al., 1978). Although not studied as extensively as IgA, IgM was completely destroyed by heating at 62.5°C for 30 min (Goldsmith et al., 1983), whereas the results of Liebhaber et al. (1977) indicated 50% retention of IgM under the same
conditions. IgG retention at 62.5°C for 30 min was reported as 86% (Goldsmith et al., 1983) and 66% (Evans et al., 1978).

Interestingly, heating milk at higher temperatures has been considered seriously detrimental to the IgA levels based on the anti-endotoxin IgA activity of milk heated at 70°C for 10, 20, and 30 min (Gaffin et al., 1983). However, since then, it has been shown that at high pasteurization temperatures, decimal reduction time (D) values for inactivation of pathogenic bacteria are much lower (fraction of a second), compared to D values required at low temperature pasteurization (Lin et al., 1987). In view of that, adequate pasteurization at high temperatures would involve heating for only a few seconds, which may not be as detrimental. In fact, D values and Z values (temperature change to alter the inactivation or degradation rate by a factor of 10) for several bacteria, lactoferrin, IgA and vitamins show that thermal processes are more detrimental to microorganisms, and that bacteria are more sensitive to temperature changes than nutrients or other quality factors present in human milk (Morgan et al., 1986; Eitenmiller, 1991). Therefore, HTST treatments should be relatively less deleterious to milk quality than bulk heating at lower temperatures which requires long treatment times. Unfortunately, there is no commercially available apparatus for effective HTST pasteurization of small volumes. This could in part explain the paucity of literature in this area. Goldblum et al. (1984), who reported the effects of heating at 72°C for 1, 3 and 15 sec and 87°C for 1, 3 and 15 sec on various protective factors, utilized a plate heat exchanger and injected pooled human milk
into a stream of sterile distilled water, in order to achieve sufficient volume to operate the apparatus. They reported no loss of IgA at 72°C for 15 sec, and complete loss at 87°C for 15 sec. On the other hand, Goldsmith et al. (1983) reported 36% IgA loss when human milk was heated at 72°C and held for 15 sec using a stainless steel capillary heat exchanger tube. They also reported total loss of IgM and 42% loss of IgG under the same conditions. Evans et al. (1978) reported total loss of IgG at 70°C, however they were using long heating time of 30 minutes. Clearly, more studies with shorter heating times are needed to assess the advantages of HTST treatment over longer heating time processes.

Recently, with the proliferation of microwave ovens, some nurseries have used this type of radiation either to thaw frozen milk or to warm freshly expressed, refrigerated human milk (Quan et al., 1992). Some preliminary work in this area shows that microwave heating at low settings (temperatures of 20 to 53°C) did not adversely affect total IgA; however, at high settings (temperature of 72 to 98°C), IgA activity against *E. coli* was negligible, possibly signifying denaturation of antibody (Sigman et al., 1989; Quan et al., 1992). Although microwaving at low settings did not affect total IgA, growth of *E. coli* in milk heated at very low settings (temperature controlled between 20 to 25°C) was 5 times the growth of *E. coli* in untreated human milk (Quan et al., 1992). There has been some discussion on the nonthermal effects of microwave radiation (Mudgett, 1989). However, even if it were possible to determine the exact mode of the biologic effects of microwaves, it would be difficult to regulate the dose of radiation, since location in the oven,
proximity to heated surfaces, and the variation in the load all affect the dose delivered (Brent, 1984).

2.2.4 Separation and loss of fat during mechanical infusion of human milk

The lipids in human milk provide approximately 50% of the calories (Garza et al., 1987) and are structured in a way that particularly suits the premature infants (Hamosh, 1987). Significant losses of milk fat occur during tube feeding of expressed breast milk to sick or premature infants (Greer et al., 1984; Stocks et al., 1985; Martinez et al., 1987). Since human milk is not homogenized, fat tends to separate out upon standing and adheres to the feeding tube or syringes (Schanler and Hurst, 1994) thus robbing the infant of an important source of calories. Martinez et al. (1987) reported cumulative losses of up to 47.4% of fat when expressed breast milk was infused at a slow rate, using a mechanical pump. Greer et al. (1984) showed that even more fat became unavailable (up to 50%) if tubing with residual milk was discarded after the syringe was empty. Moreover the extent of losses of fat associated substances such as fat soluble vitamins and trace minerals is still unknown. Although bovine milk is routinely homogenized commercially, using high pressure to rupture the fat globules, this method is unsuitable for processing human milk, as available equipment requires large volumes and is also very expensive.

Martinez et al. (1987) showed that fat loss during tube feeding could be prevented by ultrasonicating freshly expressed human milk before use.
Ultrasonication is associated with a phenomenon known as cavitation which involves making or breaking of microscopic bubbles (Hughes and Nyborg, 1962), and could be responsible for rupturing the fat globule and forming a stable suspension. Effects of ultrasonic homogenization have been shown to persist even after frozen storage for up to 4 months (Dhar et al., 1995). Additionally, feeding of ultrasonically homogenized human milk to premature neonates has been shown to result in significantly better weight gain and tricep skin-fold thickness than the control group given untreated human milk (Rayol et al., 1993). The application of ultrasonic homogenization for prevention of fat separation and loss during tube feeding is a relatively new approach, hence the literature is scant. Since feeding of sonicated human milk shows great promise in terms of the health and well being of premature neonates, more studies in this area are clearly needed. Studying the effects of sonication on milk quality, as well as improving the efficiency of the sonication procedure would be some other areas to explore.

2.3 Gram-negative bacterial infections

Infections by gram-negative bacteria are a serious and increasing problem. Gastrointestinal diseases due to gram-negative bacterial infections are responsible for high rates of morbidity and mortality in infants and young children, especially from developing countries (Guerrant et al., 1990; Echeverria et al., 1994; Black, 1993; Fomsgaard, 1990). In newborns, severe infections have been reported to result in neonatal sepsis and meningitis which is still associated with a
mortality rate of over 50% (Bonadio et al., 1991). Gram negative bacteria such as *Escherichia coli* and *Klebsiella pneumoniae* have been often associated with outbreaks of necrotizing enterocolitis in neonates (Bennet et al., 1986; Scheifele, 1990), which in turn is associated with a number of disorders from feeding intolerance to diarrhea, intestinal gangrene, perforation, sepsis and shock (Buescher, 1994).

Septicemia (gram-negative bacterial sepsis and shock) is a leading cause of morbidity and mortality among hospitalized patients. There are approximately 400,000 cases each year in the United States, and the incidence continues to rise (MMWR, 1990). Despite the availability of potent antibiotics, the administration of vasoactive drugs using advanced monitoring techniques and intensive care, the mortality rate of gram-negative bacterial shock remains distressingly high (60-80%; Ziegler at al., 1982; Bryan et al., 1983), and the situation is further complicated in immunocompromised individuals or premature infants with an underdeveloped immune system.

Since advances in antimicrobial therapy have not shown any profound impact on the lethality of septic shock, other preventative approaches, such as passive immunization using anti-lipopolysaccharide antibodies, were successfully employed during the past decade (Hammarstrom et al., 1994). In fact, the use of anti-LPS antibodies in the diagnosis and evaluation of chronic gram negative infection was also strongly suggested by Fomsgaard (1990).
There is ample experimental and clinical evidence that the endotoxin of gram-negative bacteria is one of the most important factors in the development of severe septicemia and shock (Ryan, 1985). The lipopolysaccharide molecule has numerous biological activities and is the factor responsible for many of the pathophysiological activities that accompany gram-negative infections (Rietschel et al., 1982; Ryan, 1985).

2.3.1 Lipopolysaccharide (LPS)

Lipopolysaccharides are one of the main components of the outer membrane of the cell envelope of gram-negative bacteria (Knirel and Kochetkov, 1993; Joiner, 1992). Since it is in close contact with membrane proteins, it provides membrane integrity and stability and makes possible proper functioning of the membrane (selective permeability, recognition, and intracellular interactions). As surface structures, lipopolysaccharides play an important role in the interaction of gram-negative bacteria with higher organisms. Thus, the host's defense system recognizes invading bacteria by their LPS structures and reacts with them forming anti-bacterial antibodies which are directed against determinants embedded in the LPS molecule. Endotoxins, a term often used synonymously with LPS, therefore represent immunoreactive surface antigens (Rietschel et al., 1987).

A full LPS molecule consists of a polysaccharide chain composed of repeating oligosaccharide units, a core oligosaccharide complex and a hydrophobic part called lipid A (Rietschel et al., 1982; Knirel and Kochetkov 1993). Such LPS structure (S-form) is characteristic of the majority of naturally occurring
wild strains of bacteria which form smooth colonies. Among these are Enterobacteriaceae, Pseudomonadaceae, Vibrionaceae, Brucella etc. In contrast, microorganisms forming rough colonies which are natural or artificial R-mutants produce LPS typically lacking the polysaccharide chain (Ra) or polysaccharide chain and parts of the core (Rb, Rc, Rd, Re). Smooth LPS preparations (S-form LPS) are heterogeneous mixtures of LPS molecules containing varying number of repeating oligosaccharide units in the polysaccharide chain, ranging from zero (R-form LPS) to 40 (Fomsgaard et al., 1990; Rietschel et al., 1982).

In the O-polysaccharide chain, the structure and the composition of the oligosaccharide units differs for various types of LPS and determines the O-serological specificity of the molecule, as well as the parent bacterial strain (Knirel and Kochetkov, 1993). The outer core region contains neutral sugars such as glucose, galactose and heptose, whereas the inner core region consists of unusual sugars such as ketodeoxyoctonate (KDO), galactosamine and glucosamine (Brade et al., 1986; Rietschel et al., 1987).

Endotoxins are not easily degraded under physiological conditions. Thus they are abundantly present in the environment and the gut of all animals including man (Fomsgaard, 1990). Natural detoxification mechanisms for endotoxins include anti-LPS Ab, macrophages, and the liver and Kupffer cells (Freudenberg and Galanos, 1988).
2.3.2 Anti-lipopolysaccharide antibodies

The use of anti-LPS antibodies in the treatment of acute gram negative bacterial infections in both animals and humans has been studied extensively. Various types of anti-LPS antibody and antisera have been studied. These include antibodies to anti-S form LPS, anti-O polysaccharide, anti-R-form LPS and anti-lipid A.

Therapeutic human antibodies to LPS can be obtained by the vaccination of volunteers (Ziegler et al., 1982; Calendra et al., 1988); they can be produced as monoclonal antibodies (Mab) (Larrick et al., 1985; Teng et al., 1985) or selected by screening for naturally occurring antibodies (Fomsgaard and Holder, 1993; Fomsgaard et al., 1989). The approach of obtaining antisera by immunization has several drawbacks. Immunization with Rc and Re mutant bacteria has been shown to produce widely varying antibody titres (1:2-1:256) and no booster effect. Furthermore, to circumvent the low immunogenicity of R-form LPS, the vaccine used consisted of killed rough bacteria; however these vaccines may produce antibodies to bacterial components other than LPS, as well as cause a non-specific increase in other antibodies (Siber et al., 1985). Nevertheless, using whole plasma from volunteers immunized with a mutant E. coli strain Ziegler et al. (1982) showed a significant reduction in the mortality rates of patients with gram-negative bacteremia. However, in a randomized, double blind, multicenter trial, an IgG preparation made from plasma of immunized volunteers was not found to be
more effective than IgG from non-immunized donors (Calendra et al., 1988). Reports of serious side effects to vaccination make it all the more difficult to use this approach especially in large scale (Rivat-Peran et al., 1983).

Fomsgaard (1990) chose naturally occurring anti-LPS Ab for the treatment of septic endotoxin shock. LPS from *E. coli Ra* and *S. minnesota* R60 (Ra) were used for screening donor's blood. Screening for anti-LPS antibody resolved the problems of uneven antibody response and the hazards involved in immunization of volunteers. Patients in septic endotoxic shock were treated with intravenous administration of the anti-LPS IgG obtained by screening. The treatment was coincident with the clearance of endotoxin, the disappearance of tumor necrosis factor and the improvement of clinical parameters. The anti-LPS IgG in high titred sera used above not only contained antibodies to different LPS substructures (lipid A and core) but also to S-LPS from a range of bacteria other than those used in the screening ELISA. Due to the heterogenous nature of the LPS molecules from S-form bacteria, it may be expected that naturally occurring antibodies would be directed against both lipid A, core determinants and O-groups. Thus screening for one of the types would also select sera containing anti-LPS Abs of other LPS substructure specificities. Potential problems with this approach therefore are the choice of antibody specificity and the uncertainty as to whether the anti-endotoxic effect is correlated to the anti-LPS Ab concentration. However, in an LPS-sensitive mouse model, and using the above described high titred sera, Fomsgaard (1990) was able to demonstrate that IgG anti-LPS Ab did indeed protect against lethality.
induced by LPS. Protection was not obtained against all LPS tested. By absorption experiments using LPS immobilized on a Sepharose matrix, they showed that protection was due to anti-LPS Ab specific for the particular LPS used.

Human monoclonal antibodies have also been produced against various structures of LPS (Teng et al., 1985). The use of Mab may be helpful in identifying the antigenic site(s) to which antibodies must bind in order to be protective. However the disadvantage with this approach is that the separation, purification and storage procedures are more elaborate and tedious than those for preparation of polyclonal antibodies. The cost of producing human Mab is also almost prohibitive (Hammarstrom et al., 1994).

Antibodies to LPS have also been raised and tested for possible protection against lethal gram-negative infections in animals. Bhattacharjee et al. (1994) raised antibodies in rabbits by immunization with the heat-killed J5 mutant of Escherichia coli O111 (Rc chemotype). Serum antibodies were separated into purified IgG and IgM by sequential affinity chromatography on protein G-Sepharose and anti-rabbit IgG-Sepharose columns. J5 lipopolysaccharide (LPS)-specific IgG was prepared by affinity chromatography of purified IgG on a J5 LPS-EAH Sepharose 4B affinity column. Infusing purified antibody fraction at the onset of fever (i.e., as treatment) in the neutropenic rats resulted in significant protection from lethal gram-negative bacterial infection. Tyler et al. (1990, 1991) used the same approach for raising antibodies in calves, but used LPS from Ra mutant of S. typhimurium TV119 for affinity purification by immobilizing on a
Sepharose 6B column. The purified anti-LPS immunoglobulins showed marked reactivity against a variety of gram-negative pathogens recovered from the mammary glands of cattle with clinical mastitis. The authors suggested a broad application of immunoglobulins recovered after immunization with rough mutants in prevention of coliform mastitis in cows.

Apter et al. (1993) used hybridoma cell lines for producing monoclonal secretory immunoglobulin A (slgA) against the lipopolysaccharide of *Vibrio cholerae*. A single oral dose of 5 to 50 μg of the monoclonal anti-LPS slgA, given within 2 hours before *V. cholerae* challenge, protected neonatal mice against challenge. Anti-LPS slgA was much more effective than anti-CT (cholera toxin) IgA in prevention of *V. cholerae*-induced diarrheal disease (Apter et al., 1993).

Because of the ubiquitous presence of endotoxins in nature, and especially in the gut, it is not surprising that anti-LPS Ab are present in human milk. In fact Losso et al., (1993) using LPS-ELISA showed that human milk from unimmunized lactating mothers contained substantial antibody activity against LPS of five pathogenic bacteria. The role of human milk immunoglobulins in the protection of infants against various diseases has been discussed previously, however there is lack of information on the therapeutic potential of anti-LPS Ab from human milk. Since availability of blood donors for screening and the cost of such an operation on large scale is still a big hurdle in the use of human sera, the use of human milk and colostrum for the preparation of antibodies against cell wall structures of gram-negative pathogenic bacteria could be used as an alternative. Since human
milk banks have been set up in several Children's Hospital's, around the world, obtaining donor human milk for high risk infants should not be a very difficult task.

Presence of LPS specific antibody activity has also been measured in raw bovine milk from unimmunized cows (Losso et al., 1993). Several studies have shown the usefulness of bovine immunoglobulins, from immunized cows in effectively protecting against gram-negative bacterial challenges (Mietens and Keinhorst, 1979; Tacket et al., 1988; Davidson et al., 1989)

Over the years, interest has emerged in the application of specific antibodies for the treatment of specific disease conditions. As research progresses, the role of specific antibodies in disease prevention and their mechanisms of action will become increasingly understood. One natural system that has been extensively but not thoroughly studied is the specific antibody system of human milk.

2.4 Specific antibody in human milk

Much has been written in the past two decades about the anti-infectious properties of human milk (Goldman and Smith, 1973; Pickering and Kohl, 1986; Goldman, 1993). In the past 10 years, an association has been made between the feeding or content of specific antibody in human milk and the occurrence of symptomatic infections with Campylobacter, Vibrio cholera, E. coli and Shigella.
For example, Glass et al. (1983) showed that the decreased incidence of symptoms of cholera in breastfed infants correlated with the presence of significantly higher levels of IgA antibodies to the cholera toxin and lipopolysaccharide in human milk. However, no relationship existed between antibody levels and colonization. Cruz et al. (1988) found that among infants living in a poor urban area of Guatemala and infected with enterotoxigenic *E. coli* (ETEC), those who became sick were consuming milk with significantly lower titer of antibody to heat labile toxin of ETEC compared to the infants who remained asymptomatic. In Mexico City, diarrhea owing to *Campylobacter* occurred much less frequently in breast fed compared to nonbreastfed infants (Ruiz-Pallacios et al., 1990). Furthermore, anti-*Campylobacter* sIgA was found in breast milk of all mothers except those whose infants developed *Campylobacter* diarrhea despite breast feeding. Hayani et al. (1992), also studying a Mexican population, examined milk from mothers whose infants had symptomatic or asymptomatic infections with *Shigella*. The titre of *Shigella*-specific antibodies was eight to ten times higher in milk from mothers whose infants were asymptomatic than in milk from mothers of symptomatic infants, which suggested a relationship between low specific antibody titre in milk and symptomatic disease.

Attempts have been made to increase the levels of specific antibodies in mothers milk by ingestion of either B subunit-killed whole cell or a killed whole cell oral cholera vaccine (Clemens et al., 1990). The plausibility of such a relationship originated from the known "homing" activity of the immunocompetent cells which
originate in intestinal mucosa and ultimately reside in several secretory sites including mammary epithelium, which thereby provide these sites with the ability to secrete antibodies against immunogens which are originally presented to the intestinal mucosa (Hanson, 1982). The vaccines however did not elicit significant rises of anti-LPS or anti-CT IgA breast milk antibodies (Clemens et al., 1990). On the other hand, Stoll et al. (1986) found that antibody titers to cholera toxin in milk increased in 80 to 90% of Bangladeshi patients following natural infections.

Human milk has also been shown to contain antibodies to certain viruses. Okamoto and Ogra (1989) found that following a community outbreak of respiratory syncytial virus (RSV), there was a highly significant increase in the RSV-specific IgA activity in the milk and nasopharynx, which further suggests a bronchomammary pathway in the development of virus specific antibody response in milk. Secretory IgA antibodies to rotavirus have also been detected in mothers milk (McLean and Holmes, 1980).

Specific slgA antibody profile of human milk therefore reflects the pathogens encountered by that community. This phenomenon enables human milk to provide its widely known passive protection to infants against gastrointestinal and respiratory infections. Interestingly, the continuing presence of O antigen specific slgA antibodies to diarrhoeagenic E. coli in the milk of Asian immigrant women living in Birmingham (U.K.) for more than 5 years, strongly suggests the possibility of immunological memory in the human secretory immune system (Nathavitharana et al., 1994). The titre of specific antibody in Asian
women did not correlate with their duration of stay in the United Kingdom. The highest titer of specific antibody in the group of subjects was demonstrated in a woman, in the group, who had lived in the United Kingdom for the longest period (16 years). The underlying mechanisms responsible for the continuing sIgA response in milk several years after antigen encounter needs further study and may have important implications for the development of enteric vaccines in the future.
Chapter 3

Microwave heating for rapid batch pasteurization of human milk
3.1 Introduction

Human milk is usually contaminated from the moment it is expressed. The contaminating microorganisms are mostly skin flora and occasionally pathogenic microorganisms (Lin et al., 1988). If milk is not fed to the infant immediately upon expression and is stored for some time before being fed, then depending on the type and length of storage the number of bacteria in the milk may increase and sometimes new bacteria may be found due to post expression contamination. Although conventional heating of milk is practiced in human milk banks for pasteurization of donor milk to eliminate microbes, the idea of using microwaves for pasteurization of small individual milk samples is very alluring.

A microwave oven would be a convenient in-home means to pasteurize milk and the unique heating mechanisms of microwaves could create rapid temperature increases within the material without the normal thermal lag which results from conventional heat transfer techniques (Schiffman Associates, Inc., 1989). Although several researchers have studied the effectiveness of microwaves in reducing the bacterial load of cow’s milk (Chiu et al., 1984; Knutson et al., 1988) and human milk (Sigman et al., 1989; Quan et al., 1992), a reliable method for microwaving small volumes of milk has yet not been developed. The objective of this investigation was to study the rate of heating in small volumes of water (60 mL) in a standard 2450 MHz oven with the goal of developing a protocol for possible future application to pasteurization of human milk.
3.2 Materials and methods

3.2.1 Microwave heating system

A standard, off the shelf, household microwave oven operating at 2450 MHz (Model # JE435-1, Cameo Inc., Orangeville, ON) was adapted for studying rate of heating small volumes of water. The microwave frequency used in this study (2450 MHz) is the most commonly used in North American microwave ovens (Schlegel, 1992), having a maximum penetration depth of approximately 10 centimeters (Minett, 1976). Since in-bottle heating was the objective, the bottle material selected was plastic (high density polyethylene bottles, Colgate Medical Ltd., Calgary, AB). These bottles are used regularly in milk banks and can withstand temperatures up to 100°C, and can also be stored at freezing temperatures of -20°C, which is the temperature usually recommended for long term storage of human milk (Schanler and Hurst, 1994).

A preliminary experiment was done to select from (a) tall and narrow bottle (height 125mm, depth 32mm, width 50mm) or (b) short and wide bottle (height 75mm, depth 42mm, width 55mm). Two fluoroptic probes (model # 755, Multichannel fluoroptic thermometer, Luxtron™, Mountain View, CA) were inserted in the bottle and positioned at the left and right sides (distance from sides ~5 mm; distance from surface for probe 1 ~25 mm and for probe 2 ~45 mm) for recording temperature increases. The probes were inserted through small holes made in the lid of the bottle. Since the electromagnetic field inside the microwave oven can
cause a lot of noise in some electrical measuring devices (e.g. thermocouples), interference with data acquisition when working with a microwave oven can become a problem. For this reason, a luxtron fluoroptic thermometer (not susceptible to electrical noise), was used, which enabled us to monitor and report temperature changes per second from within the microwave cavity.

The rate of heating 60 mL of water to 72°C in the microwave oven was studied in the following three setups:

3.2.2 Microwave oven with inner water bath

Surface heating has been reported for cylindrical objects with diameters in the range of 40 - 50 mm (Ohlsson & Risman, 1978). Since the bottles being used had diameters around 50 mm, surface heating patterns were expected which in turn could cause the outside portion of the milk to be overheated before the center reached desired temperature. To avoid this phenomenon, the milk bottle to be pasteurized was submerged in a water bath that would allow for even distribution of heat within the contents of the bottle. A schematic of the design is shown in Figure 1. The temperatures at three different locations within the bottle was monitored every second using fluoroptic temperature probes (model # 755, Multichannel fluoroptic thermometer, Luxtron™, Mountain View, CA) and simultaneously recorded by the computer. In order to check any difference in the heating rate between the center and the surface, the center probe was inserted deeper in the sample (~ 45 mm; total depth was ~62 mm), whereas the side probes were positioned ~ 15 mm deep from the surface of the sample, and ~5mm from the edge of the bottle.
Figure 1. A schematic of the microwave oven equipped with an inner water bath.
3.2.3 Microwave oven with agitation device

As shown in Figure 2, an external agitating device was used in order to provide faster heating rate while ensuring uniform heating. The agitator consisted of an external variable speed motor to drive a pulley (~ 100 mm in diameter) on which a rod was mounted eccentrically. A hollow plastic cylinder was mounted to the internal end of the rod, which held the sample bottle. A water spraying system (consisting of plastic tubing: length 35 cm, outside diameter ~ 6mm, inside diameter ~ 5 mm) was also installed for providing rapid cooling by spraying the bottle on all sides with cold tap water, which was collected in the tray underneath. Due to constant agitation it was difficult to position probes at different locations for temperature monitoring. Therefore one Fluoroptic temperature probe was inserted in the test bottle to monitor the temperature rise at different agitation speeds.

3.2.4 Direct heating

This involved direct heating of 60 mL of water in the microwave oven. Fluoroptic temperature probes were inserted at three different locations in the bottle (on the sides and center, as described in section 3.2.2) to monitor temperature rise every second.
Figure 2. A schematic of the microwave oven equipped with an agitation device.
3.3 RESULTS

Based on preliminary experiments looking at the water heating pattern in a narrow and a wide shaped plastic bottle (Figure 3), the narrow bottle was selected for studying the heating patterns of water in the microwave oven since the difference in temperature of water near either side of the bottle appeared less.

3.3.1 Microwave heating patterns within a water bath

Figure 4 illustrates the pattern of heating 60 mL of water in a bottle submerged in a water bath inside the microwave. This was chosen to avoid overheating of the sample in the outside portion of the container due to surface heating phenomenon. Although the heating was uniform initially (1st 15 seconds), later on the heating differential became greater (~7°C, Figure 4) between the center and the sides as shown in Figure 4. The rate of heating was also very slow, probably due to the need of heating a large volume of water in the jacket surrounding the bottle. The center of the bottle appeared to heat slower than the sides. It took almost 150 sec for the center to reach 72°C, at which time temperature of water near the sides of the bottle were close to 80°C.

In order to increase the rate of heating, a preheated water bath (72°C±1°C) was used in the same setup instead of the water at room temperature (20°C±1°C) as was used initially. Although a considerable increase in the heating rate was noticed (40 sec to reach 72°C at the center compared to 150 sec in the previous setup) the temperature differential between the sides and the center inside the bottle increased even more (Figure 5). This further confirmed the need for an agitation device for maintaining uniform heating temperatures.
Figure 3. Microwave heating patterns of 60 mL of water in two bottles with different shapes. Probes 1 and 2 were positioned in the left and right side of the sample bottle respectively. All data points are mean values of 3 sets of experiments. The S.E. of mean was < 2.0°C.
Figure 4. Microwave heating patterns of 60 mL of water immersed in a water jacket. Tap water brought to around room temperature (20°C±1°C) was used for the water jacket. This was a typical heating pattern from a series of 10 experiments.
Figure 5. Microwave heating patterns of 60 mL of water submerged in a preheated water jacket. Temperature of water in the jacket was 72°C. This was a typical heating pattern from a series of 6 experiments.
3.3.2 Microwave heating patterns under constant agitation

Figure 6 shows heating and cooling patterns of water in the microwave oven using two different agitation speeds of 30 and 50 rpm. During the heating phase, the temperature rose almost linearly in contrast to the curvilinear temperature rise usually observed with the conventional pasteurizers, where the rate of heating depends on the temperature difference between the fluids. The cooling rate however followed a more conventional temperature decrease mode because of the dependency of this cooling system on the temperature difference between the two liquids. Use of ice water instead of tap water (used in this study) for cooling could have further increased the rate of cooling. Heating 60 mL of water from 10°C to pasteurization temperature was accomplished in sixty seconds or less. The rate of heating was slightly increased by increasing the speed of agitation as seen in Figure 6. This is most likely to be the result of more homogenous heat distribution upon more vigorous agitation. This, however, cannot be said with complete certainty since we could not obtain temperature recordings from more than one location inside the bottle due to constant movement of the probes along with the fluid movement upon agitation. This design was finally abandoned due to difficulty in mechanical maintenance and also due to the risk of microwave leakage.

3.3.3 Direct microwave heating of water

Sixty mL of water was heated to 72°C in the microwave oven without a water bath. Although total heating time of less than 40 seconds was achieved (Figure 7),
Figure 6. Heating and cooling patterns of 60 mL of water in the microwaves equipped with an agitation device and a cooling system. This was a typical heating and cooling pattern from a series of 3 experiments.
Figure 7. Direct heating of 60 mL of water in the microwave oven. Probes 1 and 3 were positioned in the left and right side of the sample bottle closer to the surface of the sample. Probe 2 was positioned deeper in the center of the bottle. This was a typical heating pattern from a series of 6 experiments.
nonuniformity in heating was evident as the water temperature monitored by the thermal sensitive fluoroptic probes positioned at three different locations inside the container was not the same.

3.4 Discussion

The rate of heating small volumes of water was studied using a standard household microwave oven operating at a frequency of 2450 MHz, with the objective of developing a protocol for future pasteurization of human milk. Different designs were studied for improving the efficiency of heating, however, due to several problems encountered during the investigation such as, nonuniform heating, inefficiency of heating larger volumes, instability and expense of the agitation device and the leakage of microwaves due to design modifications, further work in this area was stopped.

Although one of the biggest advantages of using microwaves is that the sample heats rapidly, and allows for bulk heating, (i.e. heating the inside and surface of a product at the same time) heating in a microwave oven is rarely uniform (Datta and Hu, 1992). As the duration of heating increases, the spatially varying rates of heating cause the temperature at different locations to rise at different rates and this leads to an increase in the range of temperature even in liquid systems (Datta and Hu, 1992; Datta et al., 1992). This could possibly explain why the temperature difference between two positions kept increasing over time when the sample bottle was submerged in the water bath and microwaved.
Nonuniform heating has also been reported to be affected by the penetration depth of the microwaves into the material (Schiffman Associates Inc., 1989). In fact, the greater the moisture content the shallower the microwave penetration depth, and consequently, the less uniform the heating rate throughout the product (Mudgett, 1989). The penetration depth of 2450 MHz frequency microwaves in distilled water is known to be around 2.4 cm (Schiffman Associates inc., 1989). Since the diameter of the water bath was more than 15 cm, this could be another possible reason for the uneven heating seen with the setup where the sample bottle was submerged in the waterbath. The diameter of the bottles used in the study was around 50 mm, therefore nonuniform heating due to the inadequate penetration depth is unlikely when heating the water directly without the water bath. On the other hand, the problem of surface heating which has been reported for diameters in the range of 40-50 mm (Ohlsson and Risman, 1978) could explain the temperature differential observed between the side and the centers during direct heating.

In view of the above, agitating the sample during the heating process was considered necessary to ensure uniform and efficient heating. In a study by Knutson et al., (1988), cow's milk (76 mL) was heated for 59 sec resulting in a final temperature of 71.7°C. However, in the same study, heating the same amount of milk for 60-65 sec and holding for 15 sec failed to inactivate excessive numbers of *Salmonella typhimurium*, *Escherichia coli* and *Pseudomonas fluorescens*. The authors attributed that to the cold spots, which may have occurred in the milk.
during heating (due to nonuniform heating patterns), that consequently led to survival of some bacteria. This reinforces the need for careful temperature monitoring in the microwave oven. In the same study (Knutson et al., 1988), initial and final temperature of milk were measured after shaking the milk at the end of heating. This form of temperature measurement, also observed in other studies (Chiu et al., 1984; Quan et al., 1992) may evenly distribute heat generated at the end of the heating process, but does not ensure equal temperature exposure of the entire milk sample for the entire period of heating. This once again, reinforces the need for constant temperature monitoring of the sample being heated in the microwave oven.

In conclusion, although direct batch heating of small volumes to 72°C can be performed very rapidly in the microwave oven, the problem of nonuniform heating would lead to overheating of the sides before the center gets heated to the desired temperature. Use of a water bath to prevent surface heating decreases the rate of heating considerably without providing uniform heating. Constant agitation of the sample during microwave heating may be the only way of ensuring equal temperature exposure of the entire sample.
Chapter 4

Application of thermo-ultrasonication for pasteurization and homogenization of human milk
4.1 Introduction

Human milk banks have been established in children's hospitals around the world to provide a steady supply of expressed donor human milk (EHM) to premature or sick infants or those who are intolerant to commercially available formulae. There are, however, two major technical concerns regarding feeding of EHM to premature and sick neonates, a) possibility of disease transmission (Ruff, 1994; El-Mohandes et al., 1993a) and b) problem of separation and loss of a significant amount of milk fat during tube feeding the babies (Stocks et al., 1985; Garza et al., 1987; Martinez et al., 1987).

In the present study, a combination of ultrasonic waves and heat was employed to study the possibility of simultaneous pasteurization and homogenization of human milk.

Ultrasonication alone has been known to have bactericidal effects (Elliott and Winder, 1955; Davies, 1959; Hughes and Nyborg, 1962), however the combined effect of ultrasonication and heat (thermo-ultrasonication) greatly increased the bactericidal efficiency of the sonication process (Ordonez et al., 1987; Garcia et al., 1989; Wrigley and Llorca, 1992). Ultrasonication effectively homogenizes human milk fat (Martinez et al., 1987), which when fed to premature neonates resulted in significantly increased weight gain (Rayol et al., 1993). However, using the same parameters, sonication failed to kill bacteria in the absence of higher temperatures (Martinez et al., 1992). Although the process of ultrasonication itself results in some heat generation, depending on the power and the size of the disrupter horn used
(Berliner, 1984), some amount of external heat appears to be critical for increasing the bactericidal properties of ultrasonication (Ordonez et al., 1984, 1987).

The objective, in the present study, was to find those parameters of sonication (power levels, sonication amplitude, time of sonication etc.) which in combination with heat would: a) result in rapid temperature rise in the sample for quick pasteurization; b) would also homogenize the sample, without incurring major losses in immunoglobulins.

4.2 Experimental design

4.2.1 Heating profile of water and milk: effect of power level, sonicator output control settings (amplitude settings) and heat.

In the entire study samples were sonicated using the Vibra Cell, High Intensity Sonic Cell Disrupter (Sonics and Materials, Inc., Kenosia Ave, Danbury, CT 06810). Sonicators of two different power levels were studied (a) 250 watts and (b) 600 watts. Eighty mL samples of water (22°C) were sonicated directly using a 3/8th inch disrupter horn without the presence of any external heating source. Sonication was terminated when the temperature of water being sonicated reached 72°C. Temperature was monitored with a digital thermocouple (KM 450, Kane-May Ltd., Welwyn Garden City, Herts, UK).

In another experiment, eighty mL samples of human milk were sonicated at 6 different output control settings. These settings control the amplitude of vibrations at the probe tip and therefore can also be addressed as amplitude settings. The samples were sonicated directly by inserting the sonicator probe in the milk bottle which was
submerged in a hot water bath (93°C). A digitized thermocouple was used to monitor the temperature increase. Milk was also sonicated at various amplitude settings without the hot water bath and the increase in temperature monitored. A schematic of thermo-ultrasonication process is shown in Figure 8.

4.2.2 Effect of sonication amplitude on the IgA, IgG and IgM levels in human milk

This experiment was designed to study the destructive effects of amplitude of sonication process on the immunoglobulins in human milk. The purpose was to find the output control setting leading to maximum retention of all the three immunoglobulins measured. Several human milk samples were pooled and divided into 80 mL portions. These portions were subjected to sonication (3/8th inch horn, power level 600 watts) in a hot water bath. The temperature of the water bath was maintained ~93°C (boiling of water was avoided) and each sample was heated for a time that was sufficient to bring the temperature of the milk sample from 22°C to 72°C. Thereafter it was cooled in an ice bath and analysed (in triplicate) using radial immunodiffusion analysis (section 4.3.5). The time lapse between end of sonication and immersing the bottle in an ice bath was maintained at 16±1 seconds. Statistical analysis of the data was performed using one way analysis of variance.

4.2.3 Effect of sonication and thermo-ultrasonication on the IgA content of human milk over time.

This experiment was designed to study the effects of sonication (alone and in combination with heat) on IgA levels of human milk over a period of time. Here the
Figure 8. A schematic of the thermo-ultrasonication system used in this study.
output control setting of 10 was used (on a scale of 1-10), which allowed the sonicator to produce maximum energy of 600 watts/in^2. The sonicator horn used was the same as mentioned above. Three human milk samples of 160 mL each were individually divided into two 80 mL portions. One portion was sonicated (no heat) for 90 seconds and every 15 sec (from time 0 to 90 sec) a sample (25 µL) was retrieved and cooled immediately in an ice bath (3°C). The other portion was also sonicated for 90 sec (milk bottle was immersed in a water bath, 93°C) and the samples retrieved every 15 seconds and cooled as mentioned above. The samples were analysed for IgA using radial immunodiffusion analysis (section 4.3.5). Statistical comparison of retention of IgA after sonication with or without heat was performed using paired t tests.

4.2.4 Inactivation of Staphylococcus aureus in human milk by thermo-ultrasonication.

The purpose of this experiment was to determine the effect of heat and sonication, singly and in combination, on the inactivation of Staphylococcus aureus in human milk. Human milk (260 mL) was sterilized (121°C for 15 min at 15 psi) and inoculated with 10^7 CFU/ml of Staphylococcus aureus grown in trypticase soy broth. Then, 80 mL aliquots were aseptically transferred into sterile high density polyethylene containers (width 55mm, depth 42mm, height 75mm; Colgate Medical Ltd. Calgary, AB, Canada) for three different processes (Figure 9). One part was sonicated for 90 seconds, the second was "heated" without sonication for 90 seconds in an oil bath with gentle agitation (Magni-Whirl, Blue M Electric Co., Blue Island, Illinois, USA) at 173°C, and the third was sonicated while the container was submerged in the hot water bath and
Figure 9. Experimental design for inactivation of *S. aureus* in human milk by batch heating and sonication
being heated at 94°C for 90 seconds (as described previously). In all the three cases the containers were cooled after processing by immersing in an ice bath to 20°C. Once again a period of 15 to 16 sec elapsed between termination of heating and initiation of cooling. A sample of each of the processed and raw (inoculated) milk was taken for serial dilution’s (\(10^{-1} \text{ to } 10^{-8}\)), in sterile peptone water (0.1% w/v). A sample (0.1mL) of each dilution was then plated on trypticase soy agar plates (TSA, TSA+7%NaCl), using the spread plate procedure. After a few minutes the plates were incubated at 37°C (inverted position) for 24 to 48 hours. The plates were counted when the size of the colonies was big enough to be clearly seen (which was generally around 36 hours; however plates were also checked after 48 hours to see if there were any additional colonies growing).

4.2.5 Effect of sonication on homogenization of human milk

The objective here was to study the homogenization effects of the thermo-ultrasonication procedure described above, and also to compare it with other indirect forms of sonication. A flow diagram of the design is presented in Figure 10. Comparisons were also made with the Multispec M miniature homogenization system (Analytical Equipment, Coral Springs, FL). Here, the sample is first drawn into a cavity by the action of a hydraulic piston pump and on the compression stroke, pressure transferred across the homogenizer diaphragm forces the trapped milk sample through the high pressure homogenization system.
Homogenization of human milk fat

Direct

3/8" Horn
90 sec
80 mL

Multispec™
Homogenizer

Indirect

Cup horn
Water (22°C)
80 mL; 5 min

Cup horn
Water (65°C)
45 mL; 5 min

Figure 10. Experimental design for studying homogenization of human milk fat.
The indirect sonication effects were studied using a cup horn (Vibra Cell, High intensity Sonic Cell disruptor VC 600, Sonics and Materials, Inc., Danbury, CT). The cup horn screws onto the inverted converter of the sonicator. It contains a 2.5" horn and theoretically, the vibrations originating from the horn and transmitting through the water in the cup should induce cavitation inside the vessel immersed in the water (Berliner, 1984). As shown in the flow diagram, two conditions for indirect sonication using the cup horn were selected: a) immersing the milk bottle containing 80 mL of human milk into the cup horn filled with tap water (~22°C) and b) immersing the milk bottle containing 45 mL of milk into the cup horn filled with hot water (~65°C). The indirect sonication was performed for a period of 5 minutes each.

4.3 Methods

4.3.1 Sample collection

Human milk samples were obtained from British Columbia's Children's Hospital Lactation Support Service. Details on sample collection are presented in Chapter 5.

4.3.2 Microbial analysis

*Staphylococcus aureus* was enumerated using trypticase soy agar (TSA) and TSA+7% NaCl (TSAS). TSA is supposed to provide for excellent recovery of thermally injured *S. aureus* (Stiles and Clark, 1974) while TSAS suppresses growth of injured *S. aureus*. The bacteria were enumerated both before and after processing. Sufficient dilutions were performed so that plates containing 20 to 200 colonies could be
counted. Duplicate plates were counted in all cases. The spread plating technique was used where 0.1 ml of the sample, diluted $10^{-1} - 10^{-8}$, was spread on to the agar plates using a sterile glass hockey stick.

4.3.3 Source and preparation of bacterial culture

An *S. aureus* disk (ATCC 25923, Difco Laboratories, Detroit, MI) was used to inoculate 100 mL of sterile trypticase soy broth (TSB) in a 250 mL Erlenmeyer flask and incubated in a gyratory water bath (60 rpm) at 37°C for 12 hours. 10 mL inoculum of this broth containing *S. aureus* was pipetted aseptically into another flask containing 100 mL volume of TSB and incubated overnight at 37°C in the gyratory water bath (60 rpm). This procedure was repeated a third time after which 1.0 mL of this cultured broth was added to 200 μL of dimethylsulfoxide and frozen at -86°C in the Forma Scientific Enviroscan Biofreezer (model 8407, Ohio, USA). One loop of the culture was also plated on TSA to check the growth of the *S. aureus* colonies.

4.3.4 Preparation of inoculum from frozen culture

Whenever fresh *S. aureus* culture was needed, a few crystals of frozen culture were applied to the TSA plates using sterile wooden applicator sticks. Then using a sterilized loop, the crystals which had started to thaw were streaked onto the plate aseptically and *S. aureus* were allowed to grow at 37°C for 48 hours. Once the colonies were well grown, a few colonies were picked by a sterilized loop and shaken into 100 mLs of sterile TSB in a 250 mL Erlenmeyer flask and incubated at 37°C in a
shaking water bath for 12 hours. 0.1 mL of the S. aureus culture was then pipetted aseptically into another 100 mL of TSB and incubated at 37°C in a gyratory water bath (rpm 60) for 12 hours. The absorbance of this second broth was measured at 600 nm in a Shimadzu UV-Visible spectrophotometer (Tekscience, Oakville, ON), and the population determined from the standard curve of S. aureus (absorbance at 600 nm vs. log CFU/mL) prepared for this investigation. Then, depending on the desired strength of the inoculum, a calculated amount of the culture broth was aspirated using sterile pipettes and added to the milk sample.

4.3.5 Immunoglobulin analysis

Immunoglobulin levels (IgA, IgG, IgM) were quantitated using the radial immunodiffusion method of Mancini et al. (1965). The plates and standard reagents for the determination of total Ig's were obtained from Behring Diagnostics, Montreal, Quebec.

IgA analysis

For the standard, the contents of the flask containing low concentration IgA protein standard serum (LC-A) were reconstituted with 0.5 mL of distilled water. For preparing reference curves, three dilution stages (1:1, 1:3, 1:7) with isotonic sodium chloride solution were set up. 20 µL of these three different dilutions were pipetted into three corresponding wells on the LC-partigen immunodiffusion plates. The plates were placed at room temperature for 2-3 days and the diameter of the precipitation zones...
was measured and a standard curve was prepared by plotting diameter$^2$ vs. standard concentration.

A 150 fold dilution of the control serum was made using isotonic sodium chloride as diluent. 20 μL of the diluted control was applied to the plate and the diameter of the precipitation zone read after 2-3 days and values were plotted. Milk samples were diluted 8 times (1:7) with isotonic sodium chloride, and applied to the immunoplates (20 μL/well). Precipitation zones were read as above and the concentrations derived from the standard curve in mg/100 mL.

**IgG and IgM analysis**

A similar procedure was applied for IgG and IgM analysis using LC-partigen immunodiffusion plates. The standard and control dilutions used were different but the amounts injected in the plate wells were the same (20 μL). Since the concentration of IgG and IgM in the human milk samples obtained was very low, the milk samples were freeze-dried and then reconstituted with an amount of water that would increase the concentration of IgG and IgM about 4 times. These solutions were then applied to the immunoplates (20 μL/well) without diluting. Precipitation zone diameter was read as in case of IgA.

**4.3.6 Assessment of homogenization effects**

**4.3.6.1 Fat globule size determinations**

80 mL of unprocessed human milk was sonicated for 15, 30, 45, 60, 75 and 90 seconds at the sonicator output control setting of 10, using the 3/8th inch disrupter
horn (as described previously). Photomicrographs of these samples before and after sonication were taken using a microscope camera (MC 100) attached to a Zeiss phase contrast microscope (Axioskop Microscope, Oberkochen, West Germany). Size of the fat globules was determined using the micrometer photograph taken through the Zeiss microscope at the same magnification as the samples. Each photomicrograph was divided into 6 sections and the second, fourth and sixth sections were selected for counting the number and diameter of every visible fat globule. Fat globule size distribution for each photomicrograph was calculated by: number of fat globule of a particular size in the three sections/total number of fat globules in the three sections.

4.3.6.2 Fat recovery

Homogenization efficiency was studied by using laboratory models simulating tube feeding in clinical practice (Martinez et al., 1987). A slow infusion system was tested which consisted of a mechanical pump (model AS-5D, Auto-syringe Inc., Hookset, New Hampshire), and a 50 mL plastic syringe (Plastipak, Becton Dickinson, Rutherford, NJ) connected to an extension tube (152 cm long, 1.8 mL capacity, Meldon Inc., Burbank, CA) and a 40.5 cm long 8 French Argyle feeding tube (Sherwood Medical, St. Louis, MO). The syringe was maintained in a horizontal position without agitation over a period of 4 hours. The volume and rate of milk to be infused was calculated based on the amount which would be received by a 1600 g newborn at a daily rate of 150 mL/kg, representing 40 mL infusate in a 4 hour period (10 mL/hour). The degree of homogenization was measured by analysing fat
concentration in the milk before and after infusions. Aliquots of milk flowing out of the tube were collected at 30 min intervals and stored frozen at -20°C for analysis of total fat within a week. Continued air infusions were performed at the end of each trial as a precaution to recover as much milk as possible from the feeding tube.

Statistical analysis of the data (difference between each time point and zero time) was done using paired t tests.

4.3.7 Measurement of fat concentrations

The method of Lucas et al.,(1987) was employed for enzymatic determination of human milk fat. In this procedure a clarification step using Triton/EDTA and incubation in a water bath at 50°C is included for the determination of glycerol released from triglycerides in human milk samples. The triglyceride kit used in this assay was obtained from Sigma Diagnostics, St. Louis, MO.

4.3.8 Statistical analysis

Statistical analysis of the data was performed using SigmaStat™ statistical software (Jandel Scientific, San Rafael, CA).
4.4 Results

4.4.1 Effect of sonication power, amplitude and heat on temperature

Figure 11 shows the effect of different power levels of the sonicator on the heating patterns. Since no external heat source was applied, the heat generated was a direct result of the friction created by the sonic waves in the liquid being sonicated. The higher powered sonicator (600 watts) was able to raise the temperature of water from 22°C to 72°C in less than 5 min compared to almost 7 min by the low powered sonicator.

Figure 12 shows the effect of different output control settings of the 600 watt sonicator on the heating efficiency in human milk. It took almost 2 min less at output control 10 to heat the same amount of milk to 72°C, compared to output control 8. Increasing the amplitude of vibrations increased the rate of heating.

Figure 13 shows the effect of combination of sonication and heat on the rate of heating 80 mL of human milk to 72°C at various output control settings. As seen earlier, the fastest rate of heating was observed at output control 10, however, the amount of time needed to reach 72°C was dramatically less. It took around 90 sec to reach 72°C using a combination of sonication and heat (Figure 13) compared to 300 sec when sonication was used alone (Figure 12). Also, as the amplitude of sonication decreased, the time required to heat and sonicate the sample to 72°C increased.
Figure 11. Effect of sonication power on the heating patterns of 80 mL of water. Temperature was the independent variable but was plotted on the y axis for ease of interpretation. Each data point is the mean of 4 experiments. The S. E. of mean values was less than the symbol size (< 6 sec).
Figure 12. Heating patterns of 80 mL of human milk at different output control settings of sonication. No external heat was used here. Temperature was the independent variable but was plotted on the y axis for ease of interpretation. Each data point is the mean of 3 experiments. The S. E. of mean values was less than the symbol size (< 4.5 sec). A 600 watt sonicator was used in this experiment.
Figure 13. Effect of different output control settings of sonication on the heating patterns of 80 mL of human milk using thermo-ultrasonication. Temperature was the independent variable but was plotted on the y axis for ease of interpretation. Each data point represents mean of 5 experiments. The S. E. of mean values was less than the symbol size (< 2 sec).
These results were verified by repeating the experiments several times.

4.4.2 Effect of sonication amplitude on IgA, IgG and IgM of human milk

Effect of several output control settings (4, 6, 8, 9 & 10) on the IgA, IgG and IgM levels in human milk was studied. Since sonication was performed in the presence of heat (as described previously) the effect on Ig's was a combined effect of both sonication and heat. Figure 14 shows that more than 80% of the IgA, more than 70% of IgG and around 39% of IgM activity could be retained even after the most rigorous sonication (highest amplitude setting of 10) and heat combination. Statistical analysis of the data using one way ANOVA suggested no significant differences (p >0.05) between the retention of Ig's at different output control settings. In this study, only two samples were sonicated at each setting due to difficulties faced in obtaining such large volumes of human milk (80 mL for each setting). However, if the number of observations at each setting was higher the chances of differences between output control settings being significant could be greater.

Figure 14 illustrates a retention between 70 - 74% of IgG at all output control settings. This is somewhat better than 58% retention of IgG reported when human milk was pasteurized at 72°C for 15 seconds (Goldsmith et al., 1983). They also reported complete loss of IgM by both conventional and HTST processes which is much worse than our losses of 61.4% at output control setting of 10, providing...
Figure 14. Effect of sonication output control settings and heat on the immunoglobulins of human milk. 80 mL of milk was sonicated at each setting (4,6,8,9,10) with simultaneous heating to 72°C. Each bar represents mean±S.E. of 2 experiments.
maximum sonication amplitude. Although no significant differences were seen in retention of Ig's at different settings, the rate of heating using different settings varied widely (Figure 13). Since the output control setting of 10 provided most rapid heating and high retention of Ig's, this setting was selected for further studies.

4.4.3 Effect of sonication and thermo-ultrasonication on the IgA content of human milk over time

Figure 15 shows the individual effects of sonication and the combined effects of sonication and heat on IgA content of human milk over time. When sonicating alone at the output control setting of 10 (for homogenization purposes alone), some trends in reduction in IgA levels was observed between 15 and 45 sec. On the other hand, when sonicating along with heat treatment the most noticeable (but not significant, p > 0.05) drop in IgA levels was seen between 75 and 90 sec. Overall, 89±2% of IgA was retained after sonication alone compared to 78±4.8% after thermo-ultrasonication at 72°C for 90 seconds. The difference in the IgA retention between the two treatment groups was not found to be significant (p > 0.05) at any time period. However, difference in IgA retention between time zero and 90 sec was significant (p<0.05) for both treatment groups (sonication and thermo-ultrasonication). Although not found significantly different, retention of IgA in milk sonicated and heated for 45 and 60 sec appears to be somewhat higher than in milk that was only sonicated for those times.

The temperature in samples heated and sonicated
Figure 15. Effect of sonication and thermo-ultrasonication (heat and sonication) on the temperatures and IgA content of human milk over time. Percent retention values are mean±S.E. of 3 experiments. * Differences between time zero and 90 sec were significant (p <0.05) for sonication alone and thermo-ultrasonication.
simultaneously at 45 and 60 sec was 53.5° and 60.5°C respectively (Figure 15). On the other hand, temperatures in samples sonicated alone for 45 and 60 sec were 33° and 36°C respectively. Furthermore at 75 sec, retention of Ig’s with both treatments was almost the same (91.05% with sonication and 91.50% with thermo-ultrasonication) despite the difference in the temperatures (38.5°C with sonication and 66.5°C with thermo-ultrasonication; Figure 15).

At higher temperatures, the vapour pressure of gas in the bubbles formed during sonication increases, which in turn decreases the tendency of the bubbles to implode thereby reducing the intensity of cavitation (Berliner, 1984). It could therefore be possible that the additional heat generated with the external heating device (water bath) during thermo-ultrasonication could be responsible for decreasing the intensity of sonication procedure thereby protecting the IgA molecule. However, this cannot be said with certainty.

4.4.4 Effect of ultrasonication and heat on the inactivation of S. aureus in human milk

Table 1 shows the individual and combined effects of heat and sonication on the destruction of S. aureus in human milk. Sonication alone had no effect on inactivating the bacteria whereas thermo-ultrasonication effectively inactivated the inoculated microorganisms. Both treatments were conducted for the same time duration (90 sec). However, the only difference that was observed was in the final temperatures of milk (41°C when sonicated alone; 72°C when sonicated and heated simultaneously). Therefore heat appears to be an important factor in destruction of microorganisms.
Table 1
Effect of heat and sonication on *S. aureus* population in human milk

<table>
<thead>
<tr>
<th></th>
<th>Before processing (CFU/mL)</th>
<th>Inactivation of microorganisms (Expressed as log values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sonic. only¹</td>
<td>Heat only</td>
</tr>
<tr>
<td>TSA</td>
<td>$1.54 \times 10^7$</td>
<td>0.0</td>
</tr>
<tr>
<td>TSA+7% NaCl</td>
<td>$1.13 \times 10^7$</td>
<td>0.0</td>
</tr>
</tbody>
</table>

TSA: Trypticase soy agar; Sonic.: Sonication; CFU: Colony forming units. ¹The number of *S. aureus* surviving after sonication alone was $1.15 \times 10^7$ and $1.42 \times 10^7$ CFU/mL on TSA and TSAS respectively.
The question of whether sonication and heat in combination are more effective than heat alone for destroying microorganisms still remains unanswered here since heat alone (oil bath at 173°C) was equally effective in inactivating the bacteria. On the other hand, it can be said with some degree of certainty that if heat is used as the only medium for inactivating bacteria in 80 mLs of milk within 90 sec, a much higher input would be needed to raise the temperature to 72°C than was needed when heat and ultrasonication were performed simultaneously.

4.4.5 Effect of sonication on homogenization of human milk

Figures 16 and 17 show the concentration of fat in the aliquots taken before and during the four hour infusion of directly and indirectly sonicated human milk. Our objective here was to achieve a stable recovery of fat in each aliquot over the four hour period (similar to the concentration of fat present in the aliquot taken before infusion).

The indirect effects of sonication seen with the cup horn (Figure 16) were very discouraging. There was a constant drop in the fat concentration over time with a sudden and large rise in the last aliquot (similar to the untreated sample), suggesting separation of fat over time in the syringe, with a high bolus of fat in the end. Paired comparisons between time zero (before infusion) and every half hour of infusion showed significant differences (p<0.05) between time zero and 4 hour fat concentrations when 80 mL of milk was sonicated in a cup horn filled with tap water. Similarly, significant differences (p<0.05) were also seen between time zero fat concentrations and 3, 3.5 and 4 hour fat concentrations when 45 mL of milk was
Figure 16. Effect of indirect sonication using the cup horn (CH) on the fat content of milk mechanically infused at a slow rate (10 mL/h). Each data point represents mean±S.E. of 3 determinations. 80;TAP refers to 80 mL of human milk immersed in the cup horn containing tapwater (~22°C). 45;HOT refers to 45 mL of human milk immersed in a cup horn containing hot water (~65°C).
sonicated in a cup horn filled with hot water. This clearly indicates that the intensity of cavitation using the cup horn was not enough to transmit into the bottle immersed in the cup horn which therefore resulted in poor homogenizing effect.

Direct homogenization using the miniature homogenizer of the Multispec™ did not show stable fat recovery upon infusion. In fact, it appeared no different than the untreated sample. Paired comparisons showed significant differences in the fat concentrations between time zero aliquots and the last few aliquots (Figure 17). Best homogenizing effects appeared to be the ones brought about by direct sonication using the 3/8" disrupter horn (Figure 17). In the latter instance none of the statistical comparisons (between time zero and every half hour of infusion) resulted in any significant differences (p >0.05) which indicates stable infusion of milk fat, due to adequate homogenization.

4.4.6 Effect of sonication on human milk fat globule size

Figure 18 clearly shows the size distribution of fat globules in untreated and sonicated (for 90 sec) human milk. These results show that the fat globule size in untreated milk ranged from <2 μm to 10 μm, with the majority of these (40%) in the 3.5 to 6.0 μm range (Table 2), whereas in sonicated milk 56.2% of the fat globules had a diameter of < 1.5 μm. Table 2 shows the effect of sonication on the size distribution of fat globules in aliquots taken every 15 seconds during sonication. A shift in the size distribution of fat globules from larger to smaller ones over the 90 second period is evident. Figure 19 shows photomicrographs of untreated and directly sonicated human milk samples with
Figure 17. Effect of direct homogenization on the fat content of milk mechanically infused at a slow rate (10 mL/h). Each data point represents mean±S.E. of 3 determinations. 80; refers to 80 mL of human milk; 90S; refers to the time of sonication which was 90 sec.
Figure 18. Effect of direct sonication using the 3/8” disrupter horn, on the fat globule size distribution in human milk.
Table 2
Effect of sonication on the fat globule size distribution (%) in human milk

<table>
<thead>
<tr>
<th>Sonication time (sec)</th>
<th>&lt;1.5 μm</th>
<th>1.5-2.5 μm</th>
<th>2.5-3.5 μm</th>
<th>3.5-6.0 μm</th>
<th>6.0-10 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.00</td>
<td>10.0</td>
<td>25.0</td>
<td>40.0</td>
<td>25.0</td>
</tr>
<tr>
<td>15</td>
<td>0.00</td>
<td>23.8</td>
<td>61.9</td>
<td>9.50</td>
<td>4.80</td>
</tr>
<tr>
<td>30</td>
<td>0.00</td>
<td>33.4</td>
<td>45.0</td>
<td>16.7</td>
<td>5.50</td>
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<td>45</td>
<td>0.00</td>
<td>42.1</td>
<td>47.4</td>
<td>10.5</td>
<td>0.00</td>
</tr>
<tr>
<td>60</td>
<td>23.8</td>
<td>61.9</td>
<td>9.50</td>
<td>4.80</td>
<td>0.00</td>
</tr>
<tr>
<td>75</td>
<td>38.1</td>
<td>59.1</td>
<td>4.50</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>90</td>
<td>56.2</td>
<td>43.8</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Figure 19. Photomicrographs showing fat globules of human milk that was (a) untreated, (b) sonicated directly for 90 sec without heat and (c) sonicated directly for 90 sec in a hot water bath. The photomicrographs were taken at 1000 times magnification.
or without heat for 90 seconds. The size of fat globules in samples sonicated with or
without heat appeared similar.

4.5 Discussion

Thermo-ultrasonication, a process that involves simultaneous heating and
ultrasonication, was introduced by Ordonez et al. (1987) and has proved to be of
potential interest in increasing the bactericidal effect of relatively high temperatures
(below 100°C) and also seems to offer a potential to reduce heat treatment intensity
needed for pasteurization (Garcia et al., 1989).

The objective of this study was to find a method for quick batch heating of
human milk to 72°C, using heat and ultrasonication simultaneously, for the possible
advantage of homogenization. Selection of the processing variables (power levels,
amplitude settings, size of disrupter horn etc.) was based on two criteria: (a) rapid heat
generation and (b) high intensity cell disruption. A sonicator with a higher power
output (up to 600 watts) was therefore selected since it was found to generate more
heat at the same output control settings than a lower powered (250 watts) sonicator
(Figure 11). During ultrasonication, the mechanical vibrations produced by the
converter and amplified by the horn are responsible for creating the phenomenon of
"gaseous cavitation", which is the making and breaking of microscopic bubbles in the
liquid (Hughes and Nyborg, 1962). The very action of cavitation is also a heat
generating process, since input of mechanical energy causes molecular motion,
thereby raising temperature (Berliner, 1984). A higher powered sonicator would
therefore feed more electrical energy to the converter for transforming into greater
mechanical energy leading to more generation of heat. Figure 20 shows the power output (in watts) at various power monitor readings for the 600 watt ultrasonic processor used in this study. It also shows the power output of the sonicator at various output control settings. Theoretically, any sonicator providing similar power output at various output control settings should provide the same sonication effects, provided the volume, viscosity, temperature of the sample and the size of the horn remain the same. The various output control settings generally control the amplitude of vibrations at the tip of the horn (Alliger, 1975) and a specific amplitude for each given output control setting is provided by the processors. Since higher amplification results in more intense cavitation action (Berliner, 1984), more heat should therefore be generated as was observed for the maximum output control setting of 10 which produced the highest heating rate (Figure 12). This highest amplitude setting was therefore selected along with an external heat source (water bath) for the most rapid increase of milk temperatures.

Some preliminary experiments were conducted to confirm negative alkaline phosphatase activity in processed human milk. However, in order to confirm the bactericidal capacity of the process, sterile human milk was inoculated with $10^7$ CFU/mL of *Staphylococcus aureus*. *S. aureus* was selected since it is considered very resistant to the ultrasonic waves (Davies, 1959) and is also occasionally found in human milk (Lucas and Roberts, 1979; Lin et al., 1988). Ultrasonication alone was not capable of inactivating the bacteria, which is not surprising since the time for ultrasonication used was only 90 sec for an 80 mL sample. On the other hand, when ultrasonication and heat were applied simultaneously to the inoculated milk for the
Figure 20. Power output chart for the 600 watt ultrasonic processor.
(source: Instruction manual, Vibra Cell VC 600 high intensity ultrasonic processor, Sonics and Materials, Danbury, CT).
same amount of time, no growth of *S. aureus* was detected, within the limits of the enumeration methodology used. Although not observed in this study, the synergistic effect of ultrasonic waves and heat has been previously observed by a few researchers (Ordonez et al., 1984, 1987; Garcia et al., 1989; Wrigley and Llorca, 1992). In one study, decimal reduction times for thermo-ultrasonication for two strains of *Bacillus subtilis* in milk were found to be substantially lower (79% and 40% for two strains) than the decimal reduction times for thermal destruction at equal temperatures (Garcia et al., 1989). Decimal reduction times for thermo-ultrasonication for the same strains suspended in water was lower by 99% and 70%. Ordenez et al. (1987) also found that *S. aureus* cells were 5-fold more resistant in milk than in buffer. One possible explanation for this could be the concentration of solids in suspension in milk, which directly affect the cavitation intensity (Berliner, 1984) and may provide a protective effect against disintegration of bacteria. Nevertheless, the synergic effects of ultrasonication and heat observed in those studies could be due to increased sensitivity of the bacterial cells to heat resulting from changes produced in the cell membranes (Shaner, 1964) and the cytoplasmic organization (Ahmed and Russell, 1975).

From the present study, thermo-ultrasonication was not more effective than thermal treatment alone but was found to be much more effective than ultrasonication alone. One possible approach of studying the effect of heat alone or in combination with sonication was to study the destruction of bacteria as a function of time, i.e. every
15 sec, 30 sec, 45 sec and so on. However, as shown in Table 3, one important factor in that approach was the different rates of heating when using heat alone (oil bath) and when using heat and sonication combination (water bath and sonication). Even though the final temperature of milk was the same (72°C) after 90 seconds in both the water bath and the oil bath, the temperature of milk treated in the two different ways would be different at 15, 30, 45, 60 sec of the treatment (Table 3). Since temperature rise would not be same at every time point, inactivation of *S. aureus* by two different treatments could probably be a result of different temperature exposures at a particular time. However, if the rate of heating was the same with both treatments, then studying inactivation of *S. aureus* as a function of time might show if sonication and heat applied simultaneously had an added effect on destruction of *S. aureus*.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Water bath (94°C) &amp; Sonication</th>
<th>Oil bath (173°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>0.0</td>
<td>0.00</td>
</tr>
<tr>
<td>35</td>
<td>13</td>
<td>26.0</td>
</tr>
<tr>
<td>45</td>
<td>29</td>
<td>42.5</td>
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<td>55</td>
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<td>79.0</td>
</tr>
<tr>
<td>72</td>
<td>90</td>
<td>90.5</td>
</tr>
</tbody>
</table>
It is also possible that sonication would have been effective if performed for a longer time, however, that would in turn increase the final temperature of the sample which would preclude us from studying the effect of sonication alone. If one, however wishes to study the effect of sonication alone on inactivation of bacteria, the sample being ultrasonicated would have to be immersed in a water jacket attached to a temperature maintaining device to prevent temperature rise in the sample due to sonication, especially when a high powered sonicator is being used. Martinez et al. (1992) showed that sonication (up to 5 min) failed to destroy *Staphylococcus epidermidis* in human milk samples unless the temperatures were allowed to rise above 54°C. Ordonez et al. (1987) showed that the D-values for *S. aureus* using ultrasonication alone were 130 min at 24°C, and using thermo-ultrasonication were 1.9 min at 54.5°C. This suggests that using ultrasonication alone for inactivation of bacteria, would require several minutes of treatment, which in turn could be detrimental to other biologically active components of human milk.

Literature regarding effects of high intensity, KH\textsubscript{Z} frequency ultrasonication on biologically active components in aqueous solutions is scarce. Therefore, the exact mechanism responsible for reducing the activity of such factors is not known and can only be speculated upon. For instance, it has been found that during preparation of cell free extracts from gram-negative bacteria using high power sonication, activity of certain enzymes in the extracts was markedly reduced even though the sonication procedure was carried out at 4°C (Bush and Singer, 1989). The loss in activity was speculated to have been caused by intracellular proteases released during sonication.
In this study, ultrasonication of milk for 90 sec both with and without heat resulted in significant destruction of IgA. The % loss of IgA seen here (22.3% with thermo-ultrasonication), was still much less compared to loss of IgA resulting from the pasteurization procedure currently being used in the human milk banks (33%, Wills et al., 1982; 49%, Eitenmiller, 1991). The destruction of IgA during thermo-ultrasonication could be attributed to heat denaturation, however the loss of IgA by sonication alone (10.9%), could only be related to other non-thermal mechanical effects of sonication. Normally, the mechanical forces caused by collapsing cavitation bubbles do not break chemical bonds, unless the molecules are over half a million molecular weight (Alliger, 1975). However, bond breaking in DNA due to nonmechanical forces such as creation of free radicals during transient cavitation can take place (Elsner and Lindbald 1989). Inactivation of enzymes during ultrasonic processing for instance has been shown to be entirely due to oxidation by free radicals (Hughes and Nyborg, 1962). On the other hand ultrasonication process has been successfully used to break antibody-virus bonds without denaturing either product (Alliger, 1975). Free radicals can be formed when the localized heat produced during bubble collapse acts upon the gas within the bubble, producing chemical changes (Alliger, 1980).

Interestingly, ultrasonication at high temperatures does not result in production of free radicals, since the increase in vapour pressure and number of molecules within the cavitation bubble has the effect of transferring the high heat produced during bubble collapse to the outside bubble surface (Flynn, 1964). Nevertheless most of
these chemical effects of ultrasonication are seen with prolonged action (Alliger, 1980). Unfortunately, most of the studies regarding effects of ultrasonics on biocomponents, has been conducted using MH\textsubscript{2} frequencies, at very low power levels, and therefore cannot be directly used to evaluate the effects observed in the present study.

During thermo-ultrasonication, the rise in temperature of milk can lead to increase in cavitation nuclei or bubble formation due to lower surface tension and higher vapour pressure (Hughes and Nyborg, 1962). However, whereas more bubbles are formed, the violence of bubble collapse is decreased since high vapour pressure inside the bubble acts as a cushion, thereby decreasing the intensity of cavitation (Berliner, 1984), which could be responsible for slightly higher retention of IgA observed at 45 and 60 sec of thermo-ultrasonication compared to sonication alone. Martinez et al. (1992) found that ultrasonating human milk at >55°C destroyed 56% of IgA and 57.5% of IgG. Although heat could be responsible for some denaturation, the long period of sonication (4 min) could have also directly contributed to the loss in activity. The 4 min period of sonication in that study was selected on the basis of adequate homogenization of human milk fat using a 1/2" disrupter horn (500 watt sonicator). In the present study, by using a 3/8" disrupter horn, known to provide more intense sonication and greater cell disruption (Instruction Manual, 1989) adequate homogenization of human milk fat for infusion purposes was achieved in 90 sec (Figure 17), with 56% of the fat globules being <1.5 \textmu m in size. Since milk was not exposed to the ultrasonication process for a long time, that could explain high
retention of IgA (78%), IgG (74%) and IgM (37.6%) seen. Processing human milk at high temperatures (72°C for 15 sec), has been previously shown to result in total loss in IgM activity and 42% loss in IgG activity (Goldsmith et al., 1983).

Although not studied here, sonication has also been known to increase antioxidant activity of milk (Taylor and Richardson, 1980) which would prevent formation of peroxides during storage and therefore prevent development of off flavours in milk. It has also been shown that sonication of human milk did not bring about an increase in the peroxide value greater than the conventional pasteurization process (Dhar, 1989), and may in fact protect against excess peroxide formation in pasteurized milk.

In conclusion, rapid batch heating of human milk using thermo-ultrasonication has several advantages:

(a) Milk can be pasteurized and homogenized simultaneously.

(b) The process results in uniform heating due to constant agitation by high intensity ultrasonic waves.

(c) The process is capable of providing high retention of human milk immunoglobulins.

(d) The processing time is only 90 sec.

(e) The design of the system is extremely simple and the whole system is relatively inexpensive compared to existing human milk pasteurizers.

(f) Individual as well as pooled human milk samples can be processed.
Chapter 5

Pasteurization efficiency of a high temperature short time processing system for human milk.
5.1 INTRODUCTION

Several researchers around the world have endorsed the use of human milk for healthy as well as high risk infants due to its unique nutritional and immunological properties (Schanler and Hurst 1994; Hamosh 1994; Ebrahim 1995). The transfer of passive immunity to the baby via human milk is extremely critical considering how vulnerable infants are to infections. Human milk, by virtue of its immunologically active factors (especially immunoglobulin A), can protect infants against several intestinal and respiratory infections (Glass et al., 1983; Michetti et al., 1991; Theodore et al., 1982).

Many childrens' hospitals have established milk banks to store and distribute donated expressed breast milk (EBM), for premature, small for gestational age and normal term infants who may be ill and cannot suckle (Canadian Pediatric Society, 1985). Provision of breast milk is also becoming an important issue in neonatal intensive care units (Lucas, 1993; El-Mohandas et al., 1993a).

Effective use of EBM in infant feeding requires proper handling, processing and storage in order to maintain its nutritional and immunological properties. One major concern involved in banking of human milk is its microbiological quality. Breast milk is seldom sterile, it contains numerous bacteria which probably originate from the infant's mouth or mother's skin (Lucas and Roberts, 1979), and if collected under unsanitary conditions may show high bacterial counts. Bacteria present in human milk include the potential pathogens *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella*.
*pneumoniae* (Davidson et al., 1979; Gavin and Ostovar, 1977; Lin et al., 1988). In view of the possibilities of bacterial contamination, some form of heat treatment is considered necessary in order to ensure microbiological safety of banked human milk (American Academy of Pediatrics Committee on Nutrition, 1980). The pasteurization process available for human milk today is batch pasteurization (heating at 62.5°C for 30 min). The commercially available batch pasteurizers not only are expensive (around $30,000), but also require large volumes (around 10 litres) of human milk to be processed at one time for economical operating. It is feasible for a large hospital to afford an Axicare™ CM85 human milk pasteurizer (Colgate Medical Ltd., Calgary, AB) however, most milk bank services operate on a small budget and therefore cannot afford to utilize this process.

The objective of this research was to study an economical, small scale processing device that would quickly and efficiently pasteurize small volumes of human milk (> 40 mL) and at the same time preserve its immune components (specifically immunoglobulins A, G and M). In many countries, pasteurization is defined as a heat treatment which inactivates a specified minimum of the alkaline phosphatase activity, e.g. 99.6% (Neilsen and Neilsen, 1988). Hence, alkaline phosphatase was used for testing adequate pasteurization of milk, followed by confirmation with microbial challenge studies. However, as an additional routine check for the process efficiency, the use of γ-glutamyl transpeptidase has been proposed. γ-Glutamyl transpeptidase (GGTP) is much more heat resistant than alkaline phosphatase (Andrews et al., 1987; Patel and Wilbey 1989) and therefore would still
be measurable at temperatures over and above pasteurization. Since maximum retention of immunoglobulins during heat treatment is critical, the assessment of GGTP activity as a possible indicator of any overpasteurization would, therefore, be beneficial.

5.2 Materials and methods

5.2.1 Sample collection

Expressed breast milk samples (EBM) were obtained from the B.C. Children's Hospital Lactation Support (breast milk) service in Vancouver. Each sample was predominantly fore milk (i.e., initial 65 mL) expressed by mothers lactating between one to three months postpartum. Cleaning of the hands and breasts of the donors was strictly observed according to the guidelines of the Children's Hospital Breast Milk Service (1985). All samples were collected from donors between 2 and 5 pm in sterile polyethylene containers using a breast pump under the supervision of the coordinator of Lactation Support service. Milk samples were kept frozen at -20°C until required for processing.

5.2.2 HTST continuous flow system

The HTST continuous flow system consists of tubular heat exchangers for heating as well as cooling. A peristaltic pump with variable speed control (Model 7014.20, Cole Parmer Co., Chicago) was used to pump human milk through stainless steel tubing #
316 (outside diameter 1.59 mm, inside diameter 1.08 mm, total length 2.36 m) which was immersed in a hot water bath. Because of limited availability of breast milk, the heated milk was not circulated in the cooling unit but was collected simultaneously in test tubes immersed in an ice bath maintained at 3°C (Figure 21). Since the heat treated milk was being pumped out in drops (due to the slow flow rate and small diameter of the heating coils) there was instant cooling of the milk in the ice bath.

The milk inlet temperature, water bath temperature and milk exit temperature were monitored using fluoroptic temperature probes (Model# 755, Multichannel Fluoroptic Thermometer, Luxtron™, Mountain View, CA). The system can be optimized by varying the milk flow rate and/or length to diameter ratio of the stainless steel tubing both of which affect the milk residence time (RT) in the system. At the three different flow rates used in this study (5.9 mL/min; 12.3 mL/min and 18.5 mL/min), milk residence time in the system was 74, 36 and 23 sec respectively. The length of the steel coil was so optimized that milk pumped in to the system would reach water bath temperatures in 3/4th of the RT, leaving 1/4th of the RT as holding time. Therefore holding time at RT 74, 36 and 23 sec would be 18.5, 9 and 5.75 sec. The system can be used to pasteurize any quantity of milk from 40 mL to any volume required.

Cleaning of the system was performed by pumping a 1% detergent solution (Liqui-nox™, Baxter Diagnostic Corporation, Canlab Division, Burnaby, B.C.) through the steel coils at 18.5 mL/min for 20 min followed by distilled water for 20 min. For sanitation, a solution of sodium hypochlorite (200 ppm) was pumped through the system at a flow rate of 18.5 mL/min, for 10 min followed by distilled water for 10 min.
Figure 21. HTST continuous flow system.
Cleaning and sanitation both were performed at water bath temperatures of 60±2°C. Sodium hypochlorite was used as a source of chlorine which is known to be active against all bacteria, including bacterial spores, and is also known to be effective over a wide range of temperatures (Barkley, 1981).

5.2.3 Milk processing

EBM was processed separately for three different experiments.

5.2.3.1 Alkaline phosphatase and immunoglobulins in heat processed milk.

EBM from 15 mothers was collected in individual bottles. Five samples were pooled giving ~300 mL of breast milk in each of the three pools. Each individual pool was pumped into the HTST system at three different water bath temperatures (71±0.2°, 72.8±0.2° and 74±0.2°C). At each processing temperature, milk was pumped at three different flow rates 5.9, 12.3 and 18.9 mL/min corresponding to holding times of 18.5, 9.0 and 5.75 sec respectively. A portion of each milk sample processed at the temperatures and flow rates mentioned above was collected in test tubes submerged in an ice bath and stored at 4°C until analysed.

Alkaline phosphatase was analysed using two methods: (a) The quality screening method for residual phophatase in milk (AOAC, 1990), using Rutager’s pasteurization kit (Applied Research Institute, Perth Amboy, NJ). This screening test detects milk that has been pasteurized to the extent equivalent to <0.1% raw milk. (b) The second method determinates alkaline phosphatase based on enzymatic
hydrolysis of p-nitrophenol phosphate yielding p-nitrophenol and inorganic phosphate. When made alkaline, p-nitrophenol is converted to a yellow complex readily measured at 400-420 nm (Bessey et al., 1946). Reagents for the assay were obtained from Sigma Diagnostics (St. Louis, MO). Immunoglobulin (Ig) levels were analysed using radial immunodiffusion technique of Mancini et al. (1965). Plates and standard reagents were obtained from Behring Diagnostics, (Montreal, Quebec). For IgA, a 20 μL milk sample diluted 1:7 with isotonic sodium chloride was added to each well. Plates were placed in the dark for two to three days according to the manufacturer's recommendations. Diameter of the precipitation zone was then measured and compared to the standard curve prepared by plotting d² of the precipitin rings versus standard concentration. IgG and IgM were similarly analysed (details given in Chapter 4). Diameter of the precipitation rings was read as in the case of IgA. Statistical comparison of retention of immunoglobulins in milk processed at different temperatures was performed using one way analysis of variance, followed by pairwise multiple comparisons using Student Newman Keuls test.

5.2.3.2 Microbial challenge tests

Pooled human milk (220 mL from 8 donors) was sterilized (at 121°C for 15 min at 15 psi) and inoculated with *Staphylococcus aureus* to give a sample containing 10⁷ CFU/mL milk. Fresh culture of the organism was prepared as follows: A S. aureus disk (ATCC 25923, Difco Laboratories, Detroit, MI) was used to inoculate 100 mL of sterile Trypticase soy broth (TSB) in a 250-mL flask and incubated in a shaking water bath
(60 rpm) at 37°C for 12 hr. A 10-mL inoculum of this broth was pipetted aseptically into another 100 mL of TSB and incubated overnight under the conditions mentioned above. This procedure was repeated a third time and subsequently the absorbance of this broth was measured at 600 nm and the concentration read from the standard curve of *S. aureus* (absorbance at 600 nm vs. Log CFU/mL). A calculated amount of the culture was then aspirated using sterile pipettes and added to the milk sample to give $10^7$ CFU of *S. aureus*/mL.

Initial temperature of milk was maintained at 5.5°C by submerging the flask containing milk in a temperature controlled water bath. An untreated sample of milk was aseptically drawn into a sterilized test tube, which served as baseline. The milk was pumped through the system at three flow rates (5.9, 12.3 and 18.9 mL/min) which corresponded to three different holding times (18.9, 9.0 and 5.75 sec). Milk was collected in sterile test tubes at the end of the heating section. A sample was collected each time the flow rate was changed and simultaneously cooled in the ice bath (3°C). Bacteria were enumerated both before and after processing. All samples were serially diluted using 0.1% (w/v) peptone water ($10^{-1}$ to $10^{-8}$). After each dilution, 0.1 mL was spread plated on trypticase soy agar (TSA) and TSA with 7% salt (TSAS) in duplicates. The plates were incubated at 37°C for 24 to 48 hr.

A similar experiment was done using *Escherichia coli* ATCC 25922 (fresh culture obtained from microbiology division, B.C. Children's Hospital, Vancouver) as the challenge organism. Human milk (240 mL) was sterilized (at 121°C for 15 min at 15 psi) and inoculated with $10^6$ CFU/mL of *E. coli*. The inoculum of *E. coli* ($10^6$
CFU/mL) was prepared using Macfarland Standard 0.5 (PML Microbiologicals, Tvalatin, OR). The milk processing, dilutions and plating were performed as described for S. aureus, except that plating was done on nutrient agar. The plates were incubated at 35°C for 24 to 48 hr.

5.2.3.3 γ-Glutamyl transpeptidase (GGTP) activity of pasteurized bovine milk.
This experiment was designed to study the effect of heating bovine milk at different temperatures in the HTST pasteurizer on the γ-glutamyl transpeptidase activity as a possible indicator for the extent of pasteurization. Milk samples (n=7) were processed at six different temperatures (65°, 67°, 69°, 71°, 73° and 75°C). Holding time at each temperature was 18.5 sec which corresponded to a flow rate of 5.9 mL/min. The samples were cooled simultaneously in an ice bath following heating and then analysed for GGTP using the method of Naftalin et al. (1969). Reagents for the assay were obtained from Sigma Diagnostics (St. Louis, MO).
5.3 Results and discussion

Temperatures for studying human milk pasteurization were selected based on the results of Table 4 which shows the alkaline phosphatase activity in cow's milk processed at various water bath temperatures ranging from 65\(^\circ\) to 74\(^\circ\)C in the continuous flow HTST pasteurizer. Temperatures above 68.5\(^\circ\)C resulted in negative alkaline phosphatase activity. Table 5 shows the alkaline phosphatase activity in raw and heat treated human milk. Heat treatment at all temperatures (71.0\(^\circ\), 72.8\(^\circ\) and 74.0\(^\circ\)C) and flow rates (5.9, 12.3 and 18.9 mL/min) resulted in negative alkaline phosphatase activity in milk using both procedures of AOAC (1990) and Bessey et al. (1946). Testing of alkaline phosphatase as an efficient pasteurization marker has long been a statutory assay for bovine milk (Sanders and Sager, 1948; Richardson, 1985).

In some parts of the world there are government guidelines advocating its use in human milk banks also (Department of Health and Social Security, 1981). However, to be completely certain of the effectiveness of the pasteurization process, microbial challenge tests were conducted using *E. coli* and *S. aureus* as the challenge organisms. Table 6 shows the number of organisms (CFU/mL) in human milk before and after the heat treatment. It is clear that there were no *S. aureus* and *E. coli* surviving the thermal process within the detection limits of the enumeration methodology used. Therefore, the process was capable of completely inactivating the organism *S. aureus* \((10^7 \text{ CFU/mL})\) and *E. coli* \((10^6 \text{ CFU/mL})\) whether milk was held for
Table 4.
Effect of HTST pasteurization conditions on alkaline phosphatase activity of bovine milk

<table>
<thead>
<tr>
<th>MILK</th>
<th>INITIAL T(^3) (°C) (MILK)</th>
<th>WATER BATH T (°C)</th>
<th>FLOW RATE ml/min</th>
<th>EXIT T (°C) (MILK)</th>
<th>ALKALINE PHOSPHATASE (P/NP)(^1)</th>
<th>(IU/L)(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAW</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NP</td>
<td>283±27</td>
</tr>
<tr>
<td>HEATED</td>
<td>5.5</td>
<td>65.1</td>
<td>5.9</td>
<td>65.0</td>
<td>NP</td>
<td>60.5±7.1</td>
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<td>12.3</td>
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\(^{1}\) Pasteurized (P)/Non-Pasteurized (NP) milk; screening for residual phosphatase using Rutager's pasteurization kit.

\(^{2}\) International Units of Alk. Phosphatase/Litre using the modified procedure of Bessey et al. (1946).

\(^{3}\) Temperature (T). Values for alkaline phosphatase are mean±S.E. of 5 experiments. Fluctuations in all temperatures recorded was < 0.2 °C.
Table 5

Effect of HTST Pasteurization conditions on alkaline phosphatase activity of human milk

<table>
<thead>
<tr>
<th>MILK</th>
<th>INITIAL T(^3) (°C) (MILK)</th>
<th>WATER BATH T (°C)</th>
<th>FLOW RATE mL/min</th>
<th>EXIT T (°C) (MILK)</th>
<th>ALKALINE PHOSPHATASE (P/NP)(^1)</th>
<th>(IU/L)(^2)</th>
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<tbody>
<tr>
<td>RAW</td>
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<td></td>
<td>NP</td>
<td>200±23.4</td>
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<td>5.5</td>
<td>70.9</td>
<td>5.9</td>
<td>70.5</td>
<td>P</td>
<td>0.0</td>
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<td>0.0</td>
</tr>
</tbody>
</table>

\(^1\) Pasteurized (P)/Non-Pasteurized (NP) milk; screening for residual phosphatase using Rutager's pasteurization kit.
\(^2\) International Units of Alk. Phosphatase/Litre using the modified procedure of Bessey et al. (1946).
\(^3\) Temperature (T) . The experiment was repeated 3 times (n=3). Fluctuations in all temperatures recorded was < 0.2°C.
Table 6

Inactivation of *S. aureus* and *E. coli* in human milk by heat processing at 71.0°± 0.1°C

<table>
<thead>
<tr>
<th></th>
<th>BEFORE PROCESSING (CFU/mL)</th>
<th>INACTIVATION OF MICROORGANISMS¹ (Expressed as log values)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>HOLDING TIME (sec)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18.5</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>TSA</td>
<td>1.2×10⁷</td>
</tr>
<tr>
<td></td>
<td>TSA+7% NaCl</td>
<td>1.2×10⁷</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>NA</td>
<td>1.4×10⁶</td>
</tr>
</tbody>
</table>

TSA: Trypticase soy agar; NA: Nutrient agar.

¹No growth of any organism was observed in any media. However, since the first dilution plated was 10⁻¹, zero survival of organism following processing could not be said with a certainty.
18.5 sec, 9 sec or 5.75 sec in the heating coils. Enumeration of S. aureus was done using both TSA and TSAS. TSAS has been shown to suppress heat shocked S. aureus whereas both TSA and Baird Parker medium have been shown to provide almost equal recovery of thermally injured S. aureus in human milk (Lin et al., 1987). In our study, however, we found no difference in the CFU on either TSA or TSAS following heat treatment (Table 6), which clearly ruled out the possibility of the presence of thermally injured S. aureus organisms in this case. Data regarding thermal destruction rates of S. aureus in human milk under HTST conditions is scarce. In one study, the predicted D values at 67°C for S. aureus in human milk on TSA and TSAS were 3.4 and 2.8 sec and Z values were 6.5 and 6.4°C respectively (Lin et al., 1987). At 71°C, the predicted values (based on their calculations) for S. aureus would be 0.83 sec on TSA and 0.66 sec on TSAS. Therefore, based on the data of Lin et al. (1987), the holding time of 9.0 and 18.5 sec used in our study would be sufficient to inactivate 1.25*10⁷ CFU/mL of S. aureus.

Although several researchers have studied the effect of pasteurization on E. coli in human milk (Eyres et al., 1978; Lucas and Roberts 1979; Jones et al., 1979, Baum, 1973; Morgan et al., 1988), most dealt with longer pasteurization times of 10 to 30 min. Baum (1973) reported a thermal death rate curve for E. coli in human milk pasteurized at 62.5°C for various holding times up to 15 min. Calculations based on the data of Baum (1973), indicate a D value of 190 sec for E. coli at 62.5°C. However, this value is considerably larger than 12.4 sec at 62°C, reported by Morgan et al., 1988. The discrepancy could be due to the different pasteurization techniques used
(holder vs. continuous). Nevertheless, calculations based on data of Morgan et al., (1988) suggest that the D value for *E. coli* at 71°C should be 0.028 sec. Thus the holding time of 5.75, 9 and 18.5 sec used in our study should be enough to inactivate $1.49 \times 10^6$ CFU/mL of *E. coli*.

Morgan et al. (1988) showed that preheated human milk inoculated with *E. coli* and heat treated at 62°C for 15 sec gave 14 fold higher CFUs of *E. coli* on nutrient agar than on the selective media (Voilet Red Bile agar, VRB). After treatment at 64°C for 33 sec, *E. coli* was not detected on VRB while counts averaged $1 \times 10^4$ CFU/mL on NA. The authors suggested occurrence of sublethal thermal injury with an increase in the proportion of injured cells with the increase in the severity of heat treatment. In the present study, where nutrient agar was used as the enumeration medium, results showing no growth of *E. coli* in milk heated at 71°C indicate very effective destruction of the organisms.

The heating temperature of 71°C selected for microbial challenge tests was the lowest resulting in negative alkaline phosphatase activity based on a preliminary screening where a wide range of temperatures were tested. It would be logical to assume that at temperatures higher than 71°C, the destructive effects of this process would be greater, resulting in zero survivors.

Although alkaline phosphatase is a good indicator of minimal processing, recent interest in high temperature treatments requires an enzyme system that is more heat stable and can be used for routine analysis of process efficiency. $\gamma$-Glutamyl transpeptidase has been found to be less labile than alkaline phosphatase at
temperatures above 70°C (Andrews et al., 1987). Figure 22 shows γ-glutamyl transpeptidase (GGTP) activity and alkaline phosphatase activity of bovine milk processed at various temperatures in our HTST heating system. Raw bovine milk contained 4097 ± 285 units/L of GGTP. This activity decreased to 90% at 65°C, 80% at 67°C and 62% at 69°C (Figure 22). When bovine milk was processed at 71°C, a temperature at which human milk was found to be pasteurized based on negative alkaline phosphatase test and microbial challenge tests, 30% of the original GGTP activity (1236 ± 63 units/L) still remained. A lower residual GGTP activity in bovine milk could indicate overpasteurization. Human milk is a precious commodity which is not freely available and should not be used for routine testing of the process efficiency of a particular apparatus. Therefore, a bovine milk sample of known GGTP activity could be randomly run through the system as a surrogate for human milk to check for the adequacy of pasteurization.

The preference of human milk over formula for the nutrition of sick and premature babies has already been established. However, whether milk should be fed raw or pasteurized is still a matter of considerable debate. Although heat treatment results in loss of some nutritional and antimicrobial factors (Goldsmith et al., 1983; Morgan et al., 1986; Sigman et al., 1989), the risk of immunologically vulnerable neonates and infants receiving contaminated milk would be too high to ignore. In addition, bacterial contamination of human milk has been recently shown to result in loss of immunological activity, especially IgA (Pardou et al., 1994). This loss of activity
Figure 22. Effect of heat treatment using continuous flow HTST pasteurizer, on the γ-glutamyl transpeptidase (GGTP) and alkaline phosphatase activity of bovine milk. Holding time at various temperatures was 18.5 sec. The values are Mean±S.E. of seven samples for GGTP and Mean±S.E. of five samples for alkaline phosphatase.
was attributed to the IgA protease present in the bacteria contaminating the milk. This further reinforces the need for some form of heat treatment that would inactivate the microorganisms and provide maximum retention of the immune factors in milk.

The effect of various pasteurization temperatures and flow rates on the percent retention of immunoglobulins was studied (Figure 23). When milk was pasteurized at 71°C at flow rates of 18.9 mL/min, with a holding time of 5.8 sec, 82.8% IgA, 79.1% IgG and 72.1% IgM were preserved. The retention of immunoglobulins in pasteurized human milk varied, depending on the water bath temperatures and flow rates. As expected, in most cases, the retention of Ig's was significantly lower (p <0.05) when milk was exposed to higher water bath temperatures (72.8°C and 74.0°C) and held for a longer time in the stainless steel coil.

There is considerable variation in the literature regarding retention of immunoglobulins following pasteurization. Some studies showed that heating milk at 62.5°C or 63°C for 30 min resulted in 33-34% loss of IgA (Goldsmith et al., 1983; Liebhaber et al., 1977; Wills et al., 1982). On the other hand, one report suggested no loss of IgA under the same conditions (Evans et al., 1978). Similar variability is seen with high temperature short time treatments. Goldsmith et al. (1983) found 36% loss of IgA whereas Goldblum et al. (1984) reported no loss of IgA following heat treatment at 72°C. Such variations could be due to differences in the design of the heating apparatus, conditions under which milk were held before and after pasteurization or differences in the methods of analysis used.
Figure 23. Effect of heat processing at various temperatures and flow rates using the HTST pasteurizer, on the immunoglobulins of human milk. Values are Mean±S.E. of 3 samples.
Morgan et al. (1986) generated kinetic data suitable for calculating retention of biological components during heat treatment of human milk at any time and temperature. They reported a D value of 319 sec at 72°C and a Z value of 5.5°C for IgA. Using those parameters the percent retention of IgA heat treated at 72°C for 15 sec was calculated to be 89.8% which is close to retention of 82.8% IgA reported in our system and is certainly more desirable than the 67% retention of IgA achieved with heating at 62.5°C for 30 min, a system currently used in human milk banks. Heat treatment of human milk at 72°C for 15 sec has been shown to result in 58% retention of IgG (Goldsmith et al., 1983). Although heating conditions in our system were different, retention of 57.3% IgG at 72.8°C for 9 sec (Figure 23) is very close. Goldsmith et al. (1983) reported total loss of IgM at both 62.5°C for 30 min and 72°C for 15 sec. This, however, is in contrast to our finding of 52.2% retention of IgM at 72.8°C for 9 sec. Retention of more than 50% IgM at 62.5°C was also previously reported by Liebhaber et al. (1977).

Ultimately, the aim of studies, such as the present one, is to explore heating conditions that would insure complete destruction of pathogenic microorganisms and at the same time provide maximum retention of protective factors in human milk. This study presents an HTST processing system, which can effectively pasteurize small, as well as relatively large, volumes of human milk and yet permits high retention of immunoglobulins.
Chapter 6

Separation of *E. coli* O111:B4 specific IgA from human milk using LPS immobilized affinity chromatography
6.1 Introduction

Acute infectious gastrointestinal diseases remain major causes of illness in infants and young children around the world (Yolken et al., 1992; Echeverria et al., 1994; Guerrant et al., 1990). Gram negative bacteria such as enteropathogenic *E. coli* (EPEC), among others are frequently implicated for causing severe infantile diarrhea (Levine, 1987). EPEC have been often found to be either the first, or second most important bacterial cause of diarrhea in infants (Toledo et al., 1983; Black et al., 1984). *E. coli* are also considered a common cause of gram negative bacteremia and sepsis in adults (Kreger et al., 1980) and children with immune deficiencies, urinary tract infections and gastrointestinal tract lesions (Bonadio et al., 1991; Miser et al., 1981; DuPont and Spink, 1969).

Since gastroenteritis can become a severe life threatening disease in susceptible persons such as infants, elderly, and immunocompromised; treatment and prevention of such conditions is critical. Although use of antibiotics is generally restricted to severe, chronic cases, the widespread inappropriate use of antibiotics has led to resistant organisms (Lamposana, 1992). Advances in therapy (antibiotics, vasoactive drugs, intensive care) have not shown a profound impact on either gram negative bacteremia or related lethality, since the mortality rates remain distressingly high (50-80%; Ziegler et al., 1982; Bonadio, 1991).

Passive immunization for both prevention and treatment of infections has been recommended and undertaken successfully in the past few years (Hammarstrom et al., 1994). Passive immunization for therapeutic purposes using anti-LPS antibodies
has been successfully employed in gram-negative bacteremia (Ziegler et al., 1982; Fomsgaard et al., 1989). Immunoglobulin therapies commercially available, that are approved for use, are immunoglobulin concentrates prepared from pooled human plasma (Lassiter, 1992) and are extremely expensive. These immunoglobulin preparations are not purified antibodies specific to a disease organism and therefore are required in large quantities (hundreds of mg/kg body weight) which further increases the price. There is a need for a relatively inexpensive source of immunoglobulins and a technique that would provide purified antibodies, specific to a particular disease causing microorganism or a group of related microorganisms, that are not very expensive.

In the present study, human milk which is known to contain antibody activity against several pathogenic bacteria (Losso et al., 1993) and whose role in preventing symptomatic infectious diseases has been linked to specific antibodies (Ruiz-Pallacious et al., 1990; Hayani et al., 1992) was used as the source of immunoglobulins. Lipopolysaccharide, which is an epitope common to all gram negative bacteria, was used as a capture antigen to bind and separate specific antibodies (IgA) from human milk using affinity chromatography.
6.2 Materials and methods

6.2.1 LPS immobilization

Lipopolysaccharide derived from *E. coli* O111:B4 (purchased from Sigma Chemical Co., St. Louis, MO) was covalently immobilized onto porous chitosan beads. Chitosan beads (obtained from Fuji Spinning Co., Tokyo, Japan) were washed with distilled water followed by 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.0 (coupling buffer) and drained to a moist cake. The above washings were done with at least 20 times the amount of beads. Five mL of 12.5% glutaraldehyde (w/v in coupling buffer) was added to 5 g of chitosan beads (wet weight). The beads were incubated for 12 hours at room temperature after addition of 0.03 g of sodium cyanoborohydride. The activated beads were washed with the coupling buffer and added to 5 mL solution containing LPS dissolved in coupling buffer (2 mg/mL). Sodium cyanoborohydride (0.03 g) was added and the beads incubated for 24 hours at room temperature. The beads were washed sequentially with coupling buffer (to $A_{280}$ of <0.05), distilled water, 1M NaCl and distilled water (to $A_{280}$ of <0.01). The absorbance of all the washings was measured at 280 nm. A flow diagram of the procedure is presented in Figure 24. The amount of LPS bound to the beads was calculated by subtracting the amount of LPS left in the supernatant (after 24 h of incubation) and the amount lost in the washings from the amount of LPS initially added to the chitosan beads. The concentration of LPS in the supernatant (after 24 h) and the washings was calculated from the standard curve (LPS concentration vs absorbance at 280 nm, Figure 25).
Figure 24. Flow diagram showing the procedure of LPS immobilization on chitosan beads.
Figure 25. Standard curve of the absorbance of lipopolysaccharide from *E. coli* O111:B4 at 280 nm.
6.2.2 Milk collection

Human milk, obtained from the B.C. Children's Hospital Lactation Support Service in Vancouver was kept frozen at -20°C until required. Milk was collected from donors between 2 and 5 pm in sterile polyethylene containers using a breast pump under the supervision of the coordinator of the Lactation Support Service. The donors were lactating between one and three months postpartum. Cleaning of the hands and breasts of the donors was strictly observed according to the guidelines of the Children's Hospital Breast Milk Service (Radcliffe, 1982).

6.2.3 Milk processing

Human milk was centrifuged at 15,000 g, 5°C, for 30 min to remove fat. Casein was precipitated by adjusting the skimmed milk with 1 N HCl to pH 4.6 and allowing it to stand for 1 h at 37°C. The whey was separated by centrifugation at 15,000 g at 5°C for 1 h. The pH of human milk whey was adjusted to 7.0 with 1 N NaOH.

6.2.4 Initial purification of IgA from human milk

Initial purification of IgA from human milk was performed using jacalin chromatography (Cullina and Greally, 1993). Human milk whey (10 ml) was passed through a 5 mL Jacalin-agarose column (column height 8 cm, diameter 1.6 cm) equilibrated with PBS pH 7.2, 0.1 mM CaCl₂ (Jacalin suspension, containing 4 mg protein/mL was obtained from Sigma Chemical Co., St. Louis, MO). The column (with IgA bound on it) was incubated at 4°C for 1 h before washing with PBS pH 7.2, until the A₂₈₀ was <0.01. Bound IgA was eluted with 0.8 M galactose in PBS. Fractions with absorbance >0.05
at 280 nm were pooled. The column was regenerated with 0.1 M glycine-HCl buffer, pH 2.55 and reused for separating more IgA from human milk. The eluants from 2 to 3 runs were combined and concentrated by ultrafiltration using PM 10 membrane (Amicon, Beverly, MA). The concentrated eluant containing the IgA fraction was then dialyzed against 0.01 M PBS until free of galactose. A flow diagram providing an overview of the procedure is shown in Figure 26. The purified IgA was visualized by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and the concentration of IgA in this fraction was measured using radial immunodiffusion (RID).

6.2.5 Separation of *E. coli* O111:B4 LPS specific IgA

An affinity chromatography column (Column height 8 cm, diameter 1.6 cm) was prepared with *E. coli* O111:B4 LPS bound chitosan beads. The column was equilibrated with 0.01 M PBS, 0.15 M NaCl, pH 7.4. The purified IgA, obtained by elution from the jacalin column, was cycled 3 times through the LPS-chitosan column to separate *E. coli* O111:B4 LPS specific IgA from non-*E. coli* O111:B4 LPS specific IgA. The column was then washed with PBS, pH 7.4 until the effluent at A_280 was <0.01. *E. coli* O111:B4 LPS specific IgA was then eluted from the column with 0.15 M glycine-HCl, pH 2.6. The acidic fractions were immediately neutralized with 1 M Tris-HCl, pH 8.0. The eluate containing *E. coli* O111:B4 LPS specific IgA was collected as 2 mL fractions in test tubes containing 0.1 mL of 1 M Tris-HCl, pH 8.0. Specific activity of anti-LPS IgA in the various fractions was evaluated by enzyme-linked immunosorbent assay (ELISA). A brief overview of the procedure is presented in Figure 27.
Figure 26. Flow diagram showing the procedure for preparation of IgA from human milk.

**Procedure:**
1. **Passed through Jacalin column**
2. **In 0.01 M PBS, pH 7.2**
3. **Fat removed**
4. **Casein removed**
5. **Bound IgA Eluted with 0.8 M Galactose in PBS**
6. **Concentrated by ultrafiltration**
7. **Removed galactose by dialysis**
8. **Applied to LPS-chitosan column**
LPS immobilized on chitosan beads

Equilibrated with 0.01 M PBS, 0.15 M NaCl, pH 7.4

Jacalin purified IgA passed through

Column washed with PBS

Specific IgA eluted with Gly-HCl pH 2.6

Figure 27. Flow diagram showing the procedure for separation of LPS specific IgA from jacalin purified IgA.
6.2.6 Column binding capacity

A 1 mL affinity column was prepared with LPS-bound chitosan beads. The column was equilibrated with PBS, 0.15 M NaCl, pH 7.4. Jacalin purified IgA was added to the column in individual 1 mL portions, allowed to run under gravity and collected in separate test tubes and the eluate absorbance measured at 280 nm. This process was continued until the A\textsubscript{280} of the effluent reached the A\textsubscript{280} of the IgA solution being applied, which would indicate complete saturation of the column. The column was then washed with PBS, pH 7.4 until the A\textsubscript{280} of the effluent was <0.02. Bound LPS specific IgA was then eluted with 0.15 M glycine-HCl buffer, pH 2.6, and the acidic fractions neutralized as mentioned earlier. Fractions with absorbance >0.03 were combined, dialyzed against 0.05 M PBS, pH 7.2-7.4, concentrated by lyophilization and then concentration of IgA measured using radial immunodiffusion analysis (RID). The purity of anti-LPS IgA recovered was assessed by SDS-PAGE.

6.2.7 Percentage of \textit{E. coli} O111:B4 LPS specific IgA in human milk

An LPS bound chitosan column was equilibrated with PBS, 0.15 M NaCl, pH 7.4. In order to capture all \textit{E. coli} O111:B4 specific IgA present in the sample (jacalin purified IgA), less than half of the amount of sample needed to saturate the column was applied. The sample was cycled 3 times through the column. The column was then
washed with PBS, 0.15 M NaCl, pH 7.4 until the $A_{280}$ of the effluent was $< 0.02$. The *E. coli* O111:B4 LPS specific IgA was then eluted with 0.15 M glycine-HCl, pH 2.6 and immediately neutralized as mentioned earlier. Fractions with $A_{280} > 0.03$ were combined, dialyzed against 0.05 M PBS, pH 7.2-7.4, concentrated by lyophilization and then assayed for concentration of IgA using RID.

6.2.8 Galactose determination

Galactose was measured using the modified phenol-sulfuric acid method of Saha and Brewer (1994). To 0.5 mL of sugar solution, 0.5 mL of 5% phenol solution was added and mixed. Then 2.5 mL concentrated $H_2SO_4$ was added directly to the solution within 1-2 sec. The mixture was then vortexed and allowed to stand for 30 min at room temperature. Absorbance of the orange-yellow color generated was read at 490 nm against a blank prepared by substituting distilled water for the sugar solution.

6.2.9 Protein determination

Eluant protein concentration was assessed by measuring optical density at 280 nm with a Shimadzu UV-Visible spectrophotometer (Tekscience, Oakville, ON).
6.2.10 Measurement of eluant IgA concentration

Immunoglobulin A levels were quantitated using radial immunodiffusion technique of Mancini et al. (1965). Plates and standard reagents were obtained from Behring Diagnostics (Montreal, Quebec). Standard reagent diluted to different concentrations with isotonic saline was added to the plate (20 μL/well). Eluent IgA (20 μL/well) was applied as is, or diluted 1:9, 1:19, 1:29 with isotonic saline. Plates were placed in the dark for 2 to 3 days according to the manufacturer's recommendations and diameter of the precipitation zone was then measured and standard curve prepared by plotting \( d^2 \) of the precipitin rings of the standard versus the standard concentration. Concentration of the eluant IgA was read from the standard curve.

6.2.11 Antigen binding activity of Enteropathogenic E. coli O111:B4 LPS specific IgA

Specific antibody activity of the anti-E. coli O111:B4 LPS IgA (anti-LPS IgA) separated from the LPS-chitosan column was measured using enzyme-linked immunosorbent assay (ELISA), where LPS was used as the antigen. The method of Li-Chan et al., (1994) was adopted with slight modifications. The specific activity of anti-LPS IgA was measured against LPS from E. coli O111:B4 (the capture antigen) and four other pathogenic bacteria.
Reagents

Goat anti-human IgA (alpha chain specific) alkaline phosphatase conjugate, p-nitrophenyl phosphate substrate tablets, porcine gelatin type A and LPS fractions from five different bacteria (E. coli O111:B4, E. coli O128:B12, Salmonella typhimurium, Pseudomonas aeruginosa and Klebsiella pneumoniae) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Immulon II 96-well microtitre plates were purchased from Fisher Scientific (Ottawa, ON, Canada). All other reagents were of analytical grade.

Buffers

Buffers included the carbonate coating buffer at pH 9.6 (1.59 g Na$_2$CO$_3$; 2.93 g NaHCO$_3$; 0.2 g NaN$_3$; 1 L H$_2$O), phosphate-buffered saline-Tween (PBS-Tween) at pH 7.4 (8.0 g NaCl; 0.2 g KH$_2$PO$_4$; 1.15 g Na$_2$HPO$_4$; 0.2 g KCl; 0.2 g NaN$_3$; 0.5 mL Tween 20; 1 L H$_2$O) and a 10% diethanolamine buffer (97.0 mL diethanolamine; 0.1 g MgCl$_2$; 0.6H$_2$O; 0.2 g NaN$_3$; concentrated HCl to pH 9.8; H$_2$O to 1 L).

ELISA

The 96-well microtitre plates were incubated for 18 hours at 4°C with 100 µL/well of 50 mg/mL LPS in carbonate coating buffer, pH 9.6. After washing three times with PBS, pH 7.4, the wells were incubated for 30 min with 200 µL of blocking agent (0.75%, w/v, porcine gelatin in PBS). The plates were then washed three times with PBS-Tween. The anti-LPS IgA sample was diluted with PBS-Tween and 100 µL of each dilution
added per well. After incubation for 60 min the plates were washed again three times with PBS-Tween. Then 100 μL of goat anti-human IgA alkaline phosphatase conjugate (1:10,000 in PBS-Tween) were added to each well and the plates were incubated for 60 min. Wells were washed three more times with PBS-Tween and given a final rinse with distilled water before addition of 100 μL substrate (0.5 mg/mL p-nitrophenyl phosphate in diethanolamine buffer, pH 9.8). After incubation for 30-60 min, the absorbance at 405 nm was measured with an ELISA plate reader (Model EAR 400 from SLT-Lab Instruments, Austria). The specific activity of the anti-LPS IgA separated was measured using three dilutions in triplicate, for a total of nine replicates/sample.

6.2.12 SDS-PAGE

SDS-polyacrylamide gel electrophoresis was done under reducing conditions on Pharmacia Phast System using a 12% homogeneous PhastGel and Coomassie Brilliant Blue staining according to the manufacturer's recommendations (Pharmacia Biotech. Inc., Uppsala, Sweden). The anti-LPS IgA separated from chitosan-LPS column was added to the sample buffer to give a final protein concentration of 1-3 mg/mL, 2% SDS, 0.05% bromophenol blue and 2% mercaptoethanol in 0.04 M Tris-HCl, pH 8.0 buffer. Samples were heated for 5 min at 100°C and 3 μL were applied. Pharmacia's SDS-PAGE molecular weight standards, 14.4-116 kilodaltons, (Pharmacia Biotech. Inc., Uppsala, Sweden) were used as molecular weight markers. Molecular weight determinations of proteins on the gels was done with a Pharmacia
Phast Gel analyzer according to the manufacturer's recommendations (PhastImage user's manual, 1989, Pharmacia, Biotech. Inc., Uppsala, Sweden). The gels were preserved in 10% acetic acid and 5% glycerol.

6.2.13 Limulus amoebocyte lysate assay

Limulus amoebocyte lysate (LAL) assay was used to determine leaching of LPS (endotoxin) from the LPS-chitosan column under various buffer conditions used during washing and elution procedures. Presence of endotoxin (even picogram quantities) activates the enzymes of the Limulus Amoebocyte Lysate, which then degrade the chromogenic substrate, releasing the chromophore, p-nitroaniline, which can be measured at 410 nm. A quantitative chromogenic LAL procedure (QCL-1000, Whittaker M.A. Bioproducts, Walkersville, MD) was adapted. Briefly, serial dilutions of the effluent or eluant were prepared, and 50 μL of each dilution was transferred to a pyrogen free 96 well microtitre plate heated at 37°C on a block heater. LAL solution (40 μL) was added to each well, the plate gently tapped on the side to facilitate mixing and then incubated for 10 min at 37°C. Chromogenic substrate (100 μL) was then added to each well and incubated an additional 3 min. The reaction was halted by adding 50% glacial acetic acid (100 μL) to each well. Optical density was measured at 410 nm.
6.3 Results

6.3.1 Immobilization of LPS

The amount of LPS bound to the chitosan beads was determined by subtracting the amount of LPS retrieved in the washings (following the 24 h coupling procedure) from the amount of LPS originally applied to the beads. The amount of LPS present in the coupling buffer wash, distilled water wash and 1 M NaCl wash was determined by taking absorbance of all washings at 280 nm and reading the concentration of LPS from the standard curve (concentration of LPS vs absorbance at 280 nm, Figure 25). A sample calculation of the amount of LPS bound to 5 g of chitosan beads, based on the $A_{280}$ of LPS is shown in Table 7.

A total of 3 mg of LPS was bound to 5 g of chitosan beads (i.e., 0.6 mg/mL). Increasing the length of the coupling procedure beyond 1 hour did not appear to bind significant additional amounts of LPS to the chitosan beads (using one way ANOVA between all time points), suggesting very rapid binding of LPS (Figure 28).

The stability of the LPS immobilized on the chitosan beads was determined by measuring leaching of LPS from the column under various buffer conditions (Table 8). Buffers of various ionic strengths and pH were passed through the chitosan column >10 times at room temperature and the effluent was measured for the concentration of LPS using amoebocyte lysate assay and by measuring the absorbance of the effluent
Table 7

Amount of LPS bound to the chitosan beads after 24 hours of coupling time

<table>
<thead>
<tr>
<th>Absorbance @ 280 nm</th>
<th>LPS (mg)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial amount applied</td>
<td>10.2</td>
</tr>
<tr>
<td>Amount retrieved after coupling</td>
<td></td>
</tr>
<tr>
<td>1. coupling buffer wash (40 mL)</td>
<td>1.18</td>
</tr>
<tr>
<td>2. 1st water wash (26 mL)</td>
<td>0.05</td>
</tr>
<tr>
<td>3. NaCl wash (40 mL)</td>
<td>0.00</td>
</tr>
<tr>
<td>4. 2nd water wash (40 mL)</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>7.2</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LPS bound to 5 g beads</th>
<th>10.2 - 7.2 = 3 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(0.6mg/g beads)</td>
</tr>
</tbody>
</table>

¹ concentration of LPS calculated from the standard curve (concentration of LPS vs. absorbance @ 280 nm)
Figure 28. Amount of LPS bound per gram wet weight of chitosan beads over time at room temperature. Values at each time are mean ± SD of three separate incubations.
Table 8
Measurement of leaching of LPS immobilized on chitosan column under various conditions

<table>
<thead>
<tr>
<th>Buffers tested</th>
<th>Absorbance @ 280 nm</th>
<th>Limulus Amebocyte Lysate assay¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mM Tris-Gly, pH 8.0</td>
<td>&lt; 0.01</td>
<td>&lt; 0.00001%</td>
</tr>
<tr>
<td>0.15 M Gly-HCl, pH 2.55</td>
<td>&lt; 0.01</td>
<td>&lt; 0.00001%</td>
</tr>
<tr>
<td>25 mM Tris-Gly, 2.0 M NaCl, pH 6.0</td>
<td>&lt; 0.02</td>
<td>&lt; 0.00001%</td>
</tr>
<tr>
<td>0.01 M PBS, 0.15 M NaCl, pH 7.4</td>
<td>&lt; 0.009</td>
<td>&lt; 0.00001%</td>
</tr>
<tr>
<td>0.1 M KH₂PO₄, pH 8.0</td>
<td>&lt; 0.011</td>
<td>&lt; 0.00001%</td>
</tr>
</tbody>
</table>

¹ % Endotoxin units of the amount bound on the column.
at 280 nm. Results showed presence of < 0.00001% of original LPS concentration in the effluent. The $A_{280}$ values of < 0.01 also indicated negligible leaching of LPS.

6.3.2 Separation of *E. coli* O111:B4 LPS specific IgA

This was achieved in two steps. First step was purification of IgA from human milk whey using the jacalin column. Figure 29 shows the elution profile of IgA purified from human milk using jacalin affinity chromatography. Since this purified IgA fraction was to be used as a source of *E. coli* O111:B4 LPS specific IgA, the specific activity of this fraction against LPS of *E. coli* O111:B4 was tested using ELISA.

The 1/100 dilution of the jacalin purified IgA (concentration, 1.25 mg/mL), gave an absorbance of 1.1 at 405 nm, suggesting high anti-LPS Ab activity (Figure 29). This fraction was also electrophoretically analysed for purity (Figure 30, lane 4).

The jacalin purified IgA fraction was diluted with PBS, pH 7.2 and passed through a 9 mL column packed with chitosan beads coupled with LPS. Several buffers were tested for providing a good binding environment. Some of those were, 25 mM Tris-glycine, pH 8.0; 0.1 M potassium phosphate, pH 8.0; 0.1 M Tris-HCl, 0.15 M NaCl, pH 8.0 and 0.01 M PBS, 0.15 M NaCl, pH 7.2. Both 25 mM Tris-glycine, pH 8.0 and 0.01 M PBS, 0.15 M NaCl, pH 7.2 gave similar results, however, the latter was
Figure 29. Elution profile of IgA purified from human milk using jacalin affinity chromatography. Binding buffer; PBS, pH 7.2; Elution with 0.8 M galactose in PBS. Insert shows the specific activity of pooled fractions (#2-10) against LPS of E. coli O111:B4.
Figure 30. SDS-PAGE under reduced conditions showing molecular weight marker (lane 1), standard IgA (lane 2), *E. coli* O111: B4 specific IgA separated using LPS-chitosan column (lane 3), Jacalin purified IgA (lane 4) and human milk whey (lane 5).
chosen for the final experiments since the sample being applied to the column was present in PBS and therefore an additional buffer exchange step could be avoided.

Similarly, several elution conditions were also tested including either increase in ionic strength or slight decrease in pH or a combination of the two. However, the single best elution was obtained with 0.15 M glycine-HCl, pH 2.6. Release of antibodies at such drastic reduction in pH could suggest high affinity of the antibody to the LPS. Figure 31 shows the use of this technique for purification of specific immunoglobulin recognizing LPS from a particular strain of *E. coli*. Fractions 4, 5 and 6 were characterized by low total protein (OD at 280 nm), while having high activity of *E. coli* O111:B4 LPS specific IgA (high ELISA OD). Fractions 9 and 10 on the other hand were characterized by somewhat lower activity of LPS specific antibody (ELISA OD) relative to the protein content of these fractions. It could indicate that antibodies with high specificity may not necessarily have high affinity. In addition, low ELISA OD of fractions with relatively high absorbance at 280 nm, could also be due to some inactivation of the antibody caused by low pH of the elution buffer.

Fractions with OD > 0.05 were collected, dialyzed against 0.05 M PBS, pH 7.2 and concentrated by lyophilization. Purity of this antibody was determined using SDS-PAGE under reducing conditions. Figure 30 shows the *E. coli* 0111: B4 LPS specific IgA fraction in lane 3. Molecular weight of the 3 bands picked by the PhastImage analyzer were 26.6, 68.2, and 92.0 kDa for the anti-LPS IgA (lane 3). For the standard IgA (lane 2), molecular weight for the 3 bands were similar; 25.6, 68.4 and 92.6. The
Figure 31. Elution profile measured as, absorbance at 280 nm and ELISA titer recognizing LPS from *E. coli* O111:B4 of an affinity column containing chitosan beads with covalently attached LPS from *E. coli* O111:B4.
three bands are characteristic of secretory component (MW around 90 kDa), heavy chains (MW around 60 kDa) and light chains (MW around 26 kDa) (Kerr, 1990).

6.3.3 Column binding capacity

A 1 mL column containing LPS immobilized chitosan beads was saturated with 2.88 mg of jacalin purified IgA (6 mL of IgA solution, concentration 0.48 mg/mL). The column was washed to remove all nonspecifically attached IgA and a total of 0.22 mg of anti-\textit{E. coli} O111:B4 LPS IgA was recovered from 1 mL of the chitosan column. This fraction when tested for specific activity against \textit{E. coli} O111:B4 LPS gave very high absorbance at 405 nm (0.297 ELISA OD from 1/50 dilution of 0.15 mg/mL antibody solution).

6.3.4 Percentage of \textit{E. coli} O111:B4 LPS specific IgA in the jacalin purified IgA

The percentage of anti-specific LPS IgA in the total IgA pool could be subject to extreme variation depending upon the exposure of the donor mother with the particular strain of the bacteria. Since the binding capacity measurement suggested that 1 mL of matrix could be saturated with 6 mL of IgA solution (concentration 0.48 mg/mL), therefore a 9 mL column (being used for this experiment) would be saturated with 54 mL of the same IgA solution. However, in this case saturation of all the ligand sites was not intended. Instead, the target was to apply IgA solution in the amount leading to < 50% saturation of the column thereby resulting in 100% uptake of anti-
*E.coli* O111:B4 IgA out of the total applied. Therefore only 20 mL of the same IgA solution was applied to the 9 mL column (concentration 0.48 mg/mL x 20mL = 9.6 mg IgA). A total of 1.18 mg of IgA was recovered which suggests the presence of 12.3% *E. coli* O111:B4 specific IgA in the jacalin purified IgA fraction. This experiment was repeated twice with very slight deviations (11±1.2%).

6.3.5 Cross-reactivity of anti-*E.coli* O111:B4 LPS IgA

ELISA reactivity of various bacterial LPS to *E. coli* O111:B4 specific IgA was tested and the results are shown in Table 9. Triplicates of three dilutions of the specific antibody (1/10, 1/50, 1/100) with a starting concentration of 0.59 mg/mL, were tested against LPS from *E. coli* O111:B4, *E. coli* O128:B12, *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. A very high ELISA reactivity of the antibody was seen against LPS from *E. coli* O111:B4, which was expected since the antibody was specifically separated against that LPS. The antibody was also found to be highly cross reactive against *E. coli* O128:B12 LPS and LPS from *Klebsiella pneumoniae*. LPS from *Salmonella typhimurium* did not elicit a very high reaction from the purified antibody.
Table 9
ELISA reactivity of various bacterial LPS to *E. coli* O111:B4 LPS specific IgA

<table>
<thead>
<tr>
<th>LPS form bacteria</th>
<th>Optical density (405nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> O111:B4</td>
<td>1.221±0.05</td>
</tr>
<tr>
<td><em>E. coli</em> O128:B12</td>
<td>0.744±0.04</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>0.283±0.02</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>0.533±0.03</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>0.713±0.07</td>
</tr>
</tbody>
</table>

*A 1/10th dilution of the *E. coli* O111:B4 LPS specific IgA obtained by LPS immobilized affinity chromatography was used. The numbers shown are Mean±S.D. of three replicates.*
6.4 Discussion

This study presents a technique for separation of antibody specific to a disease causing bacteria from human milk using LPS immobilized affinity chromatography. Since enteropathogenic *E. coli* illness tends to be clinically more severe than many other diarrheal infections in children (Levine, 1987), and since presence of antibody to *E. coli* has been reported in human milk obtained from donor mothers in Canada (Losso et al., 1993), LPS from *E. coli* serotype O111:B4 was chosen as the capture antigen for the affinity column. The LPS was covalently immobilized on porous chitosan beads using glutaraldehyde as the coupling agent. Total IgA from human milk whey was separated using Jacalin column and LPS specific IgA was then separated from the total IgA using the LPS immobilized affinity column. From the results of SDS-PAGE and LPS-ELISA, the anti-LPS IgA separated using the above mentioned technique was quite pure and also had high specific activity against LPS from *E. coli* O111:B4.

The chitosan beads used in this study contain a six carbon spacer with a primary amine group which was linked to the aldehyde group of glutaraldehyde with a reductive amination procedure using sodium cyanoborohydride, which results in a highly stable secondary amine (Hermanson et al., 1990). The amine containing ligand (LPS) was then linked to the other aldehyde group on the glutaraldehyde molecule using the same procedure. Ligands immobilized on glutaraldehyde activated supports are known to form stable bonds and the resulting supports have proved resistant to ligand leakage during long term usage (Taylor, 1991). The presence of a spacer on
the support matrix helps avoid steric hindrance and also controls the mobility of biomaterials (Seo and Kinemura, 1977). The stability of the immobilized LPS was confirmed upon repeated uses of the column at room temperature with negligible leaching of the ligand (Table 8).

Results from the present study indicate that one mL of the LPS-chitosan column bound 0.22 mg of anti-\textit{E. coli} O111:B4 LPS specific IgA. Measurement of column binding capacity is useful in assessing the binding efficiency of the column. Unfortunately, in studies where LPS-specific IgG has been separated from purified IgG using LPS-Sepharose affinity column, not much emphasis has been focused on the binding efficiency of the column (Bhattacharjee et al., 1994; Tyler et al., 1990).

The study showed recovery of 12.3% LPS specific IgA from the Jacalin purified IgA using the LPS-Chitosan column. This value is substantially higher than 0.8% anti-LPS IgA separated from donor plasma by polyethylene glycol precipitation (Fomsgaard and Holder, 1993). Since concentration of anti-LPS IgA in human milk varied considerably between individual donors (Losso et al., 1993), therefore one may not obtain the same % recovery of specific IgA from every milk sample. Presence of antibodies specific to certain enteric bacterial pathogens in human milk is also not surprising, since specifically sensitized lymphocytes from the mucosal lining of the gut have been known to home in the mammary tissue via blood circulation (Hanson, 1982; Nathavitharana et al., 1995). The high concentration of specific IgA in human milk could also be a function of the post partum lactational stage of the mother and could reflect constant exposure of the mother to the particular microorganism.
Information about the relative efficacies of specific slgA antibodies in protection is limited. Recently, Apter et al. (1993), studied the role of monoclonal anti-lipopolysaccharide and anti-cholera toxin slgA antibodies in protection against *V. cholerae* challenge. Oral doses of 5 to 50 μg of anti-LPS IgA given 2 h before *V. cholera* challenge, protected neonatal mice against challenge. In contrast, an oral dose of 80 μg of anti-CT IgA failed to protect against *V. cholerae* challenge. Since secretory IgA due to its unique structural properties is relatively resistant to proteolysis in the gastrointestinal tract, passive immunization via oral administration of specific IgA antibodies could be very effective.

Passive immunization for prevention or treatment of gram-negative bacterial infections reported in the literature has been attempted mainly using purified IgG concentrates prepared from human plasma (Fomsgaard, 1990; Hammarstrom et al., 1994), which is not readily available, is required in large amounts (300-400 mg/Kg body weight) and also involves the potential danger of disease transmission. Monoclonal antibodies for the treatment of gram-negative bacteremia and septic shock are also being experimented with (Ziegler et al., 1991; Di Padova et al., 1994), however, due to the high cost of the preparation, large scale use of this approach has not been recommended (Van Hout et al., 1994).

Using the technique described in this study, a cross reactive anti-LPS antibody (IgA) can be prepared from donor human milk or own mother’s milk for prophylactic/therapeutic purposes in high risk, immunocompromised infants. The technique, however, is not limited to human milk only. In fact, purified immunoglobulins
from blood or cow's milk or even cheese whey could be applied on the LPS-chitosan column for separation of LPS specific antibodies. The support matrix "chitosan beads" used in this study, is relatively inexpensive which makes this technique attractive for large scale operations.

In the present study, even though antibodies were separated against a smooth LPS, they were still found to cross react with other serotypes (E. coli O128:B12) and other gram-negative bacteria, specially, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, using LPS-ELISA (Table 9). Although the O-polysaccharide chain in the S-form LPS is supposed to provide a unique antigenic specificity to the LPS molecule, in nature, S-form LPS are present as heterogeneous mixtures which may contain varying number of repeating oligosaccharide units in the polysaccharide chain (Fomsgaard et al., 1990; Rietschel et al., 1982). They may even contain molecules of R-form lacking the polysaccharide chain, as well as those of SR-form (only one repeating unit in O-antigen) (Knirel and Kochetkov, 1993), which may account for some cross reactivity seen in this study. Microheterogeneity, due to the presence of some components in nonstoichiometric proportions, or replacement of one component by another in some molecules also exists (Knirel and Kochetkov, 1993) which may result in some cross reactivity.

Since the core structures of LPS are less variable (Rietschel et al., 1987), some investigators used R-form LPS for preparation of LPS specific IgG in order to obtain a broader spectrum antibody (Bhattacharjee et al., 1994; Tyler et al., 1990). Even though, polyclonal anti-core antibodies have shown to protect neutropenic rats
against lethal challenge with *Pseudomonas aeruginosa* (Bhattacharjee et al., 1994) effective use of polyclonal Ab to R-form LPS in treatment of gram-negative bacterial infections in humans has not yet been substantiated, and their advantage in protection against gram negative septic shock in humans has been questioned (Calendra et al., 1988; Geisman and Johnston, 1988).

Although the polyclonal IgA separated in this study was specific to *E. coli* O111:B4 serotype, the cross reactivity with other pathogenic bacteria observed makes it a more broad spectrum antibody preparation which could be beneficial as a preventative passive immunization measure, or could be used therapeutically in infections of mixed etiology. The broader specificity of polyclonal antibodies may in fact be desirable in screening for circulating gram-negative bacteria or LPS in the host tissue. In addition, polyclonal Ig therapy could even circumvent the problem of acquired drug resistance (Hammarstrom et al., 1994).

Attempts were also made to use human milk whey directly on the LPS-chitosan column, for separation of LPS specific antibodies. Figure 32 shows the SDS-PAGE profile of the fraction eluted from LPS-chitosan column (lane 4). Although there were a few bands characteristic of the secretory component, heavy and light chain of the IgA molecule (≈ 92 kDa, ≈60 kDa and ≈ 27 kDa respectively), the band ≈ 92 kDa appeared unusually strong, suggesting maybe the presence of some other human milk protein along with the secretory component. This approach however, was not very successful since some leaching of the ligand (LPS) was observed in the eluant.
In conclusion, the present study provides a technique for separation of LPS-specific IgA from human milk using an LPS-chitosan affinity column. Theoretically, any antibody (IgG, IgA or IgM) that is purified from any source, can be applied on this column for further separation of anti-LPS antibody. There is a great potential for application of this technique in preparation of specific antibodies for prevention of enteric gram-negative infections as well as in diagnosis of endotoxinemia. Since anti-LPS IgG can also be theoretically purified using this technique, there is a potential for its application in treatment of gram negative bacteremia.
Figure 32. SDS-PAGE under reduced conditions showing anti-LPS proteins eluted from LPS-chitosan column (lane 4), IgA standard (lane 2), human milk whey (lane 1), molecular weight marker (lane 3).
Chapter 7

Summary

Studies on human milk were conducted from two different aspects. One dealt with processing of human milk, more specifically (a) finding alternate forms of heat treatment than those currently available for pasteurization of human milk, and (b) studying the possibility of simultaneous homogenization and pasteurization of human milk. The second aspect dealt with developing a technique for separating antibodies from human milk specific to a pathogenic bacteria.

Important findings of the research have been summarized as follows:

1. A standard household microwave oven could be used for rapid batch heating of small volumes of milk (based on the heating rate of water). However, since the rate of heating was not uniform (difference of ~7°C between the side and center), the sample would need to be overheated for the cold spots to reach the desired temperature. Immersing the sample in a water bath to prevent surface heating did not reduce nonuniformity in heating. Constant agitation of the sample during heating was found to be the best way of ensuring uniform temperature distribution and rapid heating.
2. The use of high power, high intensity ultrasonication in combination with heat resulted in rapid heating of small batches of human milk (90 sec for an 80 mL sample). The process inactivated \( >10^6 \) CFU/mL of *Staphylococcus aureus* and provided high retention of IgA, IgG and IgM. The same process (combination of ultrasonication and heat) also adequately homogenized human milk, therefore resulting in a procedure for simultaneous pasteurization and homogenization of human milk. An obvious advantage was the absence of nonuniformity in heating due to constant agitation of milk during ultrasonication.

3. A continuous flow, high temperature short time heating system, such as described in this research could be used for adequate pasteurization of single or pooled human milk samples in volumes from as small as 20 mL to any amount. Although, pasteurization could be achieved at a system temperature of 71°C and flow rate of 18.9 mL/min based on the results of alkaline phosphatase tests and microbial challenge tests, higher temperatures or slower flow rates could be chosen for enhanced safety. Heat treatment at 71°C for various holding times, resulted in retention of 63-83% of IgA, 58-79% IgG and 49-72% IgM which are comparable to the batch pasteurization processes used in human milk banks.

4. A 30% residual activity of GGTP was found in bovine milk pasteurized at 71°C, using the HTST continuous system. Since at this temperature alkaline
phosphatase was undetectable, the percent residual concentration of GGTP in pasteurized milk could be used as an indicator of over or under pasteurization.

5. An affinity chromatography technique was developed for separation of enteropathogenic *E. coli* O111:B4 LPS specific IgA from human milk. The LPS-chitosan affinity column was stable as demonstrated by of leaching of LPS from the column effluents. The column had a binding capacity of 0.22mg of *E. coli* O111:B4 LPS specific IgA per mL. About 12% specific IgA was recovered from the jacalin purified IgA applied to the LPS-chitosan column, which was substantially higher than literature values for unimmunized human sera. Through use of LPS-ELISA, the purified specific IgA was found to be highly cross reactive with *E. coli* O128:B12, and *Klebsiella pneumoniae*. The low cost column material used (chitosan beads) makes large scale utilization of affinity chromatography technique for isolation of IgA with specific activity more practical.

Conclusions

Some conclusions drawn from this research are as follows:

1. There is a potential for use of standard household microwave oven for rapid batch heating of small volumes of milk, provided constant agitation of the sample is performed to prevent nonuniformity in heating.
2. Thermo-ultrasonication and HTST continuous flow procedures both resulted in rapid pasteurization of human milk. Both procedures were found to be capable of inactivating pathogenic bacteria in high numbers (>10^6 CFU/mL), and also resulted in good retention of immunoglobulins A, G and M. However, in situations where loss of milk fat is highly undesirable, such as during tube feeding of premature infants, the use of thermo-ultrasonication would be more appropriate since the process is capable of simultaneously homogenizing and pasteurizing the sample.

3. The thermo-ultrasonication and HTST system described in this research provide a much more economical option to the batch pasteurizers currently available for pasteurization of human milk.

4. The above mentioned techniques could be used very efficiently for homogenization or pasteurization of small individual milk samples, such as for processing of mother's milk for her own infant in special care nurseries.

5. Since loss of nutrients and antimicrobial factors of human milk is highly undesirable, use of a simple screening procedure such as analyzing percent residual activity of GGTP in a reference milk sample, randomly run through the system to check for the extent of pasteurization would be quite beneficial.
6. Finally, the specific antibody produced using the LPS-immobilized chitosan column could be potentially used in future for prevention/treatment of enteric gram-negative bacterial infections. If the technique is used to separate LPS-specific antibody of the IgG class, then it could be used as a therapeutic measure in life threatening bacteremia of single or mixed etiology in humans or even livestock. Antibody specific to LPS could also be used in screening for circulating gram negative bacteria or LPS in the host tissue. Since shortage of blood donors is foreseeable in the near future, IgG purified from bovine milk or cheese whey could be applied to the LPS-chitosan column for preparation of specific anti-LPS IgG antibodies. Further research, however, is needed to verify the above mentioned potential applications of the technique.
References


