ANTI-INVASIVE ACTIVITY OF BOVINE COLOSTRUM AND WHEY PROTEINS AGAINST *SALMONELLA* TYPHIMURIUM

by

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ABSTRACT

Bovine colostrum, non-immunoglobulin G (non-IgG) fraction, obtained after passing colostral whey through a protein G-agarose affinity chromatography column, and selected whey proteins (α-lactalbumin, β-lactoglobulin, lactoferrin and lactoperoxidase) were tested for anti-invasive activity against *Salmonella* Typhimurium SL 1344 invasion of HeLa cells.

Bovine colostrum and non-IgG fraction decreased *S.* Typhimurium invasion of cultured HeLa cells by 98 and 66% respectively. In an attempt to find the anti-invasive compound(s) in non-IgG fraction of bovine colostral whey, lactoferrin, α-lactalbumin, β-lactoglobulin and lactoperoxidase were tested for their effect on HeLa cell invasion by *S.* Typhimurium. All tested whey proteins except lactoperoxidase, at concentrations up to 10 mg mL^{-1}, significantly ($p<0.05$) reduced invasion. There was a significant linear relation ($p<0.05$) between whey protein concentration and anti-invasive activity when protein concentration ranged between 0 and 8 mg mL^{-1}. Anti-invasive activity of α-lactalbumin and β-lactoglobulin at the highest concentration used (15 mg mL^{-1}) was not significantly ($p>0.05$) different from anti-invasive activity at 8 mg mL^{-1}. Lactoferrin at 15 mg mL^{-1} had significantly higher anti-invasive activity than at 8 mg mL^{-1}. Lactoferrin showed the highest anti-invasive activity (78%) of the three whey proteins at 15 mg mL^{-1}. Whey proteins, at the concentrations used, were not cytotoxic to HeLa cells.

Random Centroid Optimization program was used in an attempt to formulate a mixture of lactoferrin, α-lactalbumin and β-lactoglobulin with improved anti-invasive activity. In comparison to individual proteins, all mixtures had lower anti-invasive activity per mg protein suggesting antagonistic interactions among the tested whey

proteins. Tested whey proteins were not bactericidal nor bacteriostatic at 10 and 15 mg mL^{-1} and did not promote aggregation of *S.* Typhimurium. Preincubation of HeLa cells with selected whey proteins and including the washing step before challenging with *S.* Typhimurium almost eliminated anti-invasive activity for β-lactoglobulin and reduced the anti-invasive activity of α-lactalbumin and lactoferrin by 40 and 50% respectively. Preincubation of *S.* Typhimurium with the tested whey proteins prior to invasion also resulted in reduced anti-invasive activity. Results suggest the potential of lactoferrin, α-lactalbumin and β-lactoglobulin for being considered as bioactive ingredients in food formulation.

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This thesis is dedicated to all my family members whose support, patience and encouragement brought me hope throughout all the ups and downs.

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List of Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
ATCC	American Type Culture Collection
CDC	Centers for Disease Control
CFA	Colonization Factor Antigen
CFU	Colony Forming Unit
CRFK	Crandell-Reese Feline Kidney
DAEC	Diffusely Adherent *Escherichia coli*
DIC	Differential interference contrast
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *Escherichia coli*
EDTA	Ethylene Diamine Tetra-acetic Acid
ELISA	Enzyme Linked Immunosorbent assay
EPEC	Enteropathogenic *Escherichia coli*
FBS	Fetal Bovine Serum
FCV	Feline calcivirus
FDA	Food and Drug Adminstration
ICMSF	International Commission on Microbiological Specifications for Foods
IgG	Immunoglobulin G
kDa	Kilo Dalton
LB	Lauria-Bertani
LPS	Lipopolysaccharide
MEK	Monkey Embryo Kidney
MEM	Minimum Essential Medium
mg	Milligram
mL	Millilitre
PBS	Phosphate Buffered Saline
PBS^{++}	Phosphate Buffered Saline containing $MgCl_2$ and $CaCl_2$
PBS^{--}	Phosphate Buffered Saline without $MgCl_2$ and $CaCl_2$
PV	Polio Virus

RCO	Random Centroid Optimization
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SPI-1	Salmonella Pathogenicity Island 1
UBC	University of British Columbia
μg	Microgram
μL	Microlitre
μm	Micrometer
UV	Ultra Violet
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 General Introduction

Today's consumers expect more from food than just hunger satiety. Changing consumer demands are stimulating utilization of more natural products and foods with special health benefits. This has opened a large market for nutraceuticals and functional foods and the trend seems to be increasing even more. The food industry is also increasingly shifting towards modifying product formulations to meet the expectations of health conscious consumers. Based on information collected from Japan, Europe, United States and Australia, nutraceuticals and functional foods are the fastest growing segment of the food industry with an estimated market value of $33 billion and an annual growth rate of 15-20% (Hilliam, 2000). In light of this fact, researchers have shown a great interest in extraction and utilization of biologically active substances (Jelen and Lutz, 1998).

Foodborne illness is estimated to affect 2 million people in Canada annually, with salmonellosis alone costing the economy in excess of $800 million (Todd, 1989). The United States Centers for Disease Control's best estimate is that there are 1.4 million salmonellosis cases annually in the United States alone (Frenzen *et al.*, 2003). Gastrointestinal diseases caused by *Salmonella* continue to represent a major threat to human health on a global scale. S. Typhimurium has been the most frequently isolated serovar in Canada and United States (Khakhria, Woodward, Johnson, and Poppe, 1997). Children and the elderly are more susceptible than healthy young adults. Infections can range from self-limiting gastroenteritis to life-threatening systemic enteric fevers to septicemia (Salyers and Whitt, 2002).

S. Typhimurium belongs to the group of microorganisms that are known to enter mammalian cells in order to induce their pathogenicity. The phenomenon is called bacterial invasion and is mediated by a number of genetic factors (Falkow, 1991). Existing knowledge and technology cannot be used to provide consumers with pathogen-free raw meat and poultry, and it is very unlikely that the eradication of *Salmonella* in domestic animals will occur in the foreseeable future (WHO, 1997).

Antibiotic therapy does not seem to be effective in un-complicated cases of salmonellosis. Also the emergence of *Salmonella* strains resistant to commonly used antibiotics has become a concern for clinicians, microbiologists and those responsible for the control of communicable disease. The issue has become an important concern for the food industry as well (WHO, 1997).

Of the potential novel, non-antibiotic treatment strategies, compounds that can prevent the initial attachment between bacteria and host cell, either by blocking the bacteria or tissue from attachment, are promising candidates (Breithaupt, 1999). There is a potential market for use of natural anti-bacterial compounds as natural preservative agents for foods or to enhance food safety from a microbiological point of view. These compounds could also be used for supplementation of infant formula or foods for the elderly and immunocompromised individuals, in order to prevent diarrheal illnesses caused by enteropathogens.

As mentioned earlier, salmonellosis remains an important issue in public health and a multidisciplinary approach is required in order to prevent or at least control these foodborne disease challenges. Since complete elimination of the

pathogen is not feasible with the current science, other approaches would be prevention and to interrupt the transmission–colonization link of the organism in human body.

For thousands of years, prior to the introduction of sulfa drugs and penicillin, physicians used bovine colostrum for medical use especially as an antibiotic (Rona, 1998). Even, today, animal scientists supplement animal neonates' food with colostrum to combat microbial infections. With this in mind any attempt to explore the anti-bacterial activity of this early human food seems reasonable, if not necessary.

According to the literature review, a majority of the research projects on the effect of milk proteins on bacteria have concentrated on anti-bacterial screening studies and mainly focused on bacterial growth inhibition. It is difficult to reach solid conclusions about anti-bacterial activity of these compounds on the basis of such studies. The growth inhibition methods generally involve comparison of colony forming units (CFU) in treated and non-treated samples and phenomena such as bacterial clumping may result in reduction of CFUs while no inhibition has actually occurred.

Some research studies involving the use of cell cultures for testing inhibition of invasion by *Escherichia coli* O157:H7 have been reported (Carbonare, Silva, Trabulsi, & Carneiro-Sampaio, 1995).

Facon (1995) reported anti-invasive activity of both IgG and non-IgG fraction of bovine colostrum against *S.* Typhimurium, *Salmonella enteritidis* and *Escherichia coli.* To delve more into the anti-invasive property of the non-IgG fraction, the present research was initiated with the focus of investigating the IgG-free fraction of

colostral whey and to identify active components. Since the present research was a continuation of the work done by Facon (1995) the conditions and procedures were adopted from the same reference.

1.2 Hypotheses:

- Non immunoglobulin G fraction of bovine colostral whey has anti-invasive activity against *S.* Typhimurium.
- Lactoferrin, α-lactalbumin, β-lactoglobulin and lactoperoxidase contribute to anti-invasive activity of non-immunoglobulin G fraction of colostral whey.
- Combination of lactoferrin, α-lactalbumin and β-lactoglobulin have increased anti-invasive activity compared to the individual proteins.
- The anti-invasive activity of lactoferrin, α-lactalbumin and β-lactoglobulin is not due to bactericidal activity.

1.3 Overview of work plan:

The current research was designed to study the non-immunoglobulin G antimicrobial proteins of bovine colostrum/milk and evaluate their anti-invasive activity against *S.* Typhimurium using HeLa cell culture. A monolayer culture model of HeLa cells was used in this study with a view to identify the active components and determine the possible antagonistic/synergistic effect of these components. The project consisted of three phases.

Phase I involved study of the effect of bovine colostrum, non-IgG fraction of colostral whey and selected bovine milk whey proteins on *S.* Typhimurium invasion of HeLa cells.

Phase II involved study of the effect of whey protein concentration on *S.* Typhimurium invasion of HeLa cells. The feasibility of formulating a mixture of whey proteins with improved anti-invasive activity per mg protein using Random Centroid Optimization program was also evaluated.

Phase III involved study of *S.* Typhimurium growth inhibition and aggregation properties caused by α-lactalbumin, β-lactoglobulin and lactoferrin. The effect of separately incubating the whey proteins with either *S.* Typhimurium suspension or HeLa cells prior to invasion assay was also investigated.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Foodborne infectious disease

Foodborne diseases continue to be a widespread and growing public health problem, both in developed and developing countries. Although foodborne disease may happen in any member of the population, the elderly, pregnant women, immunocompromised individuals such as AIDS and organ transplant patients, people with malignancies or individuals under treatment with immunosuppressive drugs are at greater risk. Around 2.1 million people, on a global scale, died from diarrheal diseases in 2000 mostly caused by contaminated food and drinking water (WHO, 2002a). The actual global incidence of foodborne disease is hard to estimate, since most foodborne diseases are sporadic and often the cases are under-reported especially in developing countries. In the United States of America, it is estimated that around 76 million cases of foodborne diseases occur annually leading to 325,000 hospitalizations and 5,000 deaths (Snowdon, Buzby and Roberts, 2002).

With the recent trend of globalization of food trade, international travel and changing life style, preventing a locally occurring foodborne outbreak from becoming wide spread seems difficult if not impossible. For example the outbreak of salmonellosis caused by a snack food produced in Israel caused illness in the home country and in at least two other countries, England and the United States (Killalea *et al.*, 1996). Also a brand of infant formula was responsible for outbreak of *Salmonella* Anatum in England and France (Threlfall *et al.,* 1998).

2.1.1 Impact on economy

Foodborne disease may severely affect the economy of the community. Considering only the medical costs and lost productivity, infectious diseases caused by major foodborne pathogens (*Salmonella, Campylobacter, Escherichia, Listeria* and *Vibrio cholerae*) in USA in 1997 cost $35 billion (WHO, 2002b). *Salmonella* accounted for US$4 billion among the foodborne infectious diseases noted above (WHO, 1997). Unfortunately, no recent Canadian inofrmation on the economic impact of salmonellosis, was found. It has been estimated that in North America and Europe each reported case of human salmonellosis costs between US$ 1000 to 1300. The average cost of outbreaks in North America and Europe range from around US$ 60,000 to more than US$ 20 million (WHO, 1997).

2.1.2 Salmonellosis

Salmonellosis, caused by *Salmonella*, is responsible for 84.5% of all foodborne disease outbreaks and remains an important common human illness, even in developed countries with well–developed food and water hygiene conditions (Ziprin and Hume, 2001). Despite the more advanced hygienic practices in developed countries, problems such as, "relative crowding in day care centers; increasing proportion of elderly people; large number of immunocompromised individuals and a high level of antibiotic resistance in hospitals" result in the spread of salmonellosis (Facon, 1995). Outbreaks have involved many types of food and a wide range of locations such as restaurants, institutions, cafeterias, geriatric nursing homes,

childcare facilities, hospitals and airlines. The magnitude of incidents of salmonellosis resulted in formation of a global network surveillance on *Salmonella* in January 2000 within the World Health Organization (WHO). The network has a broad spectrum of members including institutions and individuals in human health, veterinary and food-related disciplines (WHO, 2003).

2.1.2.1 Epidemiology and transmission

The annual number of cases of salmonellosis in the U.S. is estimated to be between 2 to 4 million (FDA, 2003). Salmonellosis is more prevalent in children and the elderly and it has been reported that these age groups represent 60% of all confirmed cases. Immunocompromised individuals are also at higher risk. It is estimated that the incidence of salmonellosis in AIDS patients is 20-fold higher than the general population (FDA, 2003). Many incidents of salmonellosis involve large outbreaks, but studies show that more than 80% of all salmonellosis incidents involve individual cases (WHO, 1997).

While *Salmonella* infection is considered severe but not fatal, in any large outbreak, death may occur due to development of the systemic form of the disease (Salyers and Whitt, 2002a). The US Centers for Disease Control estimates that approximately 1,000 persons die each year in the USA due to acute salmonellosis (CDC, 2001). *Salmonella* was responsible for 52% of the foodborne disease incidents and 81% of deaths attributed to foodborne disease outbreaks in nursing homes (Gray

and Fedorka-Cray, 2002). The fatality rate of typhoid fever is 10% whereas it is 1% for other forms of salmonellosis. Stage of the infection and the age group have a dramatic effect on the mortality rate (FDA, 2003).

Salmonella, which live in the intestinal tracts of humans and other animals, including birds, are usually transmitted to humans eating foods contaminated with animal feces. Although most salmonellosis cases in humans are thought to be due to consumption of raw or undercooked contaminated food of animal origin (FDA, 2003), outbreaks caused by plant foods, such as alfalfa sprouts, cantaloupe and fruit juices are on the rise (Food Safety Network, 2003). *Salmonella* can be transmitted either directly through food or through cross-contamination especially in food-service establishments and institutions such as hospitals (WHO, 1997). *Salmonella* infections can occur at any site in the body. Normally *Salmonella* enters via the oral route and invade the ileum and from there the bacteria may travel and infect other sites. Sometimes it causes infections in very unusual sites, such as the skull. Chronic carriage poses a significant public health threat, especially when food handlers or people working in health care institutions develop the carrier state (Ziprin and Hume, 2001). In the carrier state, the patient will shed the bacteria in the feces for more than 1 year (Salyers and Whitt, 2002a).

2.1.3 Salmonella

Daniel E. Salmon, U.S. veterinary surgeon, discovered *Salmonella* in 1886 (Bell and Kyriakides, 2002). To date, there are more than 2400 known serovars of

Salmonella (Gray and Fedorka-Cray, 2002). *Salmonella* is a Gram-negative, rod-shaped, nonsporeforming and motile bacterium (with the exceptions of *S.* Gallinarum and *S.* Pullorum that are not motile). *Salmonella* belong to the family of Enterobacteriaceae and are capable of growing in a wide range of laboratory media from the simple to complex. *Salmonella* are widespread in animals, especially poultry and swine. Environmental sources of the organism include water, soil, insects, factory surfaces, kitchen surfaces, animal feces, raw meats, raw poultry, and raw seafood, to name only a few. Growth has been reported over the temperature range of 7-46°C, with the optimum growth temperature between 35-37°C (FDA, 2003). *Salmonella* survive at pH 4, whereas they die off rapidly at pH 2-3 (Ziprin and Hume, 2001).

Taxonomy of *Salmonella* is a persistent challenge amongst microbiologists (Gray and Fedorka-Cray, 2002). As a result of DNA analysis, it is believed that all *Salmonella* serotypes belong to a single species called *enterica*. According to this taxonomy all members of the nontyphoidal salmonellae are designated as serovars of the *Salmonella* subspecies *enterica* and may be written as S. (italicized) followed by the serovar (non-italicized). For example, *Salmonella enterica* subspecies *enterica* serovar *typhimurium* is written as *S.* Typhimurium (Le Minor and Popoff, 1987). In the old classification scheme, *S.* Typhimurium was considered as a subgroup of *Salmonella enteritidis* and would be designated as *S. enteritidis var. typhimurium* or to abbreviate, *S. typhimurium*. The taxonomy used throughout this thesis to refer to this microorganism is *S.* Typhimurium.

2.1.3.1 S. Typhimurium

S. Typhimurium, a human and animal pathogen, is one of the most important *Salmonella* serovars transmitted from animals to humans (Gray and Fedorka-Cray, 2002). Together with *Salmonella* Enteritidis, it is responsible for the majority of the reported cases of salmonella infections. *S.* Typhimurium can be found in a broad range of animal species as well as the environment (WHO, 1997). In a study of 74 consecutive cases of salmonellosis, *S.* Typhimurium was the most common serovar causing septicaemia (Ziprin and Hume, 2001). *S.* Typhimurium infections are more frequently fatal during the neonatal period and in the presence of an associated disease such as congenital heart disorders or immunodeficiency disorders (Ziprin and Hume, 2001).

2.1.3.2 Clinical symptoms of salmonellosis

The clinical symptoms of human salmonellosis include acute fever, abdominal pain, diarrhoea, headache, nausea, and in some cases, vomiting may also occur (WHO, 1997). The symptoms may appear in 6-48 hours depending on the infective dose, *Salmonella* serovar and individual immunity. The severity of symptoms may differ from one person to another. Acute symptoms may last for 1 to 2 days or longer, depending on host factors, ingested dose, and serovar. Even as few as 15-20 cells, depending upon age and health of host, and serovar differences among the members of the genus may cause symptoms of salmonellosis (FDA, 2003). In general, due to

natural immunity in healthy individuals, the infective dose will be higher and ranges from 10^6 to 10^9 cells for most *Salmonella* serotypes (Finlay, 1994).

Salmonellosis may lead to several systemic problems such as Reiter's syndrome and rheumatoid syndromes (Adams and Motarjemi, 1999). Symptoms of chronic conditions may follow 3-4 weeks after the acute symptoms (FDA, 2003). More than 2% of the 170,000-200,000 infected people, in an outbreak of salmonellosis in Chicago caused by contaminated pasteurized milk, developed reactive arthritis (Archer and Young, 1988). Reiter's syndrome, another disease associated with salmonellosis, is speculated to be caused by abnormal immune reaction to the infectious agent and thought to be a combination of urethritis, conjunctivitis and arthritis (Ziprin and Hume, 2001).

Another problem associated with salmonellosis, especially in the very young and in the elderly is dehydration due to severe diarrhea, which can become life threatening (WHO, 1997). Prolonged diarrhea leads to nutrient deficiency and consequently malnutrition. Diarrhea is a major cause of malnutrition in infants and young children (WHO, 2002a). In developing countries, diarrheal diseases, particularly infant diarrhea, are a major public health problem. It has been estimated that some 1.5 billion children under five years of age suffer from diarrhea and as a result over 3 million die annually (Adams and Motarjemi, 1999).

2.1.3.3 Treatment

S. Typhimurium gastroenteritis is normally self-limiting and usually it takes 5-7 days to resolve. Treatment is recommended only when the patient becomes severely dehydrated due to excessive diarrhea or in case the infection spreads from the intestines. Persons with severe diarrhea may require rehydration, often with intravenous fluids. There is no evidence that administration of antibiotics shortens the duration of symptoms (Salyers and Whitt, 2002a). Thus, antibiotic therapy is not recommended for uncomplicated cases of gastroenteritis and even when it is applied in severe cases, the antibiotic should be used within 48 hours of the disease onset (Wiström and Norrby, 1995). Also antibiotic therapy has been reported to prolong the excretion of *Salmonella* (Aserkoff and Bennett, 1969). Antibiotic treatment does not make a difference in whether or not the person develops arthritis (CDC, 2001). Since the early 1990s, antibiotic resistant strains of *S.* Typhimurium have emerged and are increasingly threatening public health as more antibiotics are rendered ineffective. The use of antibiotics in intensive animal husbandry is considered a possible explanation for the emergence of resistant bacteria (WHO, 1997). *S.* Typhimurium DT 104 is one of these resistant bacteria (Gray and Fedorka-Cray, 2002). The organism is resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracyclines. The prevalence of *S.* Typhimurium isolates with the five-drug pattern of resistance increased from 0.6% in 1979-1980 to 34% in 1996 (Ziprin and Hume, 2001).

Development of vaccines to treat salmonellosis has been slow (Ziprin and Hume, 2001) and is mainly focused on typhoid fever. There are currently at least 2 vaccines against typhoid fever on the market for use in underdeveloped countries. The vaccines are mostly used for livestock in developed countries. The duration of conferring immunity is not known yet and repeated vaccination may cause systemic side effects. Orally administrated vaccines seem to be more promising (Ziprin and Hume, 2001).

In more complicated situations treatment may involve surgical removal of the affected area (Ziprin and Hume, 2001). Chronic asymptomatic carrier state of *Salmonella* after recovery from acute symptomatic salmonellosis is often hard to treat. Appropriate treatment for both the acute and asymptomatic carrier state is still unclear.

2.2 Bacterial pathogenicity

The stage in which the body is colonized by disease-causing-bacteria, is called infection and when the infection produces symptoms it proceeds to disease. Pathogenicity is the ability of the bacteria to cause disease. Pathogens cause disease by producing enterotoxins, by damaging intestinal microvilli or by invading cells of the intestinal epithelium (Salyers and Whitt, 2002b).

2.2.1 Bacterial invasion

Invasion following the adherence of the bacteria to the host cell is an important bacterial virulence factor (Alwan, Deignan, O'Sullivan, Kelly and O'Farrelly, 1998). The invasion process is different amongst invasive bacteria and is regulated by bacterial proteins. Briefly, these proteins cause actin rearrangement in the host cells that are not normally phagocytic. This phenomenon may lead to formation of pseudopods, which engulf the bacteria in a phagocytic vacuole (Salyers and Whitt, 2002a).

S. Typhimurium needs to invade intestinal epithelium to cause disease (Finlay, 1994; Zierler and Galán, 1996). Intestinal colonization by *S.* Typhimurium is not enough for development of disease and penetration of epithelial cells is necessary (Gray and Fedorka-Cray, 2002). Although it has been reported that *S.* Typhimurium produces small amounts of cholera-like toxin (Formal, Hale and Sansonetti, 1983) the role of this toxin in disease development is not clear and may serve as secondary pathogenicity. Studies with non-invasive mutants of *S.* Typhimurium showed that those bacteria lack the ability to cause gastroenteritis in humans (Johnston, Pegues, Huek, Lee and Miller, 1996; Penheiter, Mathur, Giles, Fahlen and Jones, 1997). On the other hand, non-invasive mutants of *S.* Typhimurium were rendered invasive once the genes for invasion were inserted and expressed (Galán and Curtiss, 1989).

Salmonella invasion is distinct from receptor-mediated phagocytosis and it seems that *Salmonella* has a specific mechanism for entering the epithelial cells and forces its uptake by host cells. Although the mechanism of the entry and signalling of

Salmonella is different depending on host cells, it has been proven that regardless of the host cell type, *Salmonella* may initiate membrane ruffling and forced engulfment in any type of eukaryotic cell (Galán, 1996). The invasion of *Salmonella* into cells is initiated by the bacteria at the cell surface and involves a complex signalling procedure. The speculated route is through M cells, which are located over Peyer's patches (Finlay, 1994; Galán, 1996). M cells have a thinner mucus layer, shorter microvilli and have active endocytic and pinocytic uptake compared to columnar epithelial cells. These differentiated cells are the pathways through which many pathogens gain access to deeper tissues (Finlay and Falkow, 1997). Upon contact with epithelial cells, secretion of a series of proteins is stimulated and special surface appendages, invasomes, are formed on *S.* Typhimurium (Zierler and Galán, 1995) which disappear immediately after the bacteria are internalized. Invosome structure does not develop when *S.* Typhimurium is grown in broth. The cell contact seems necessary for this phenomenon (Finlay and Falkow, 1997).

A cluster of chromosomal genes called *Salmonella* Pathogenicity Island-1 (SPI-1) is responsible for conferring invasiveness to the bacteria. This region contains more than 28 genes, which in turn encode the bacterial type III secretion system that is required for virulence of *Salmonella* and many other gram negative pathogens (Collazo and Galán, 1997; Goosney, Knoechel, and Finlay, 1999; Brumell, Steele-Mortimer, and Finlay, 1999). During the invasion process, *Salmonella* utilize this secretion system to release proteins that enter the host cell and apparently interact with unknown host cell components. The molecular mechanism of the type III secretion system export pathway is not still clear. It seems that, following the contact with host

cells, *Salmonella* injects the secreted molecules into the host cells (Finlay and Falkow, 1997). The genes necessary for invasion were reviewed by Galán (1996) and are designated as *inv* (A-J). The importance and role of each gene is different as characterized by gene disruption analysis (Salyers and Whitt, 2002a). *S.* Typhimurium also triggers calcium and inositol fluxes. Chelating agents block the entry of the *S.* Typhimurium into the cultured cells (Finlay and Falkow, 1997). Subsequently, these interactions induce alterations in the actin cytoskeleton followed by membrane ruffling and force the pinocytosis of the bacteria in large vacuoles (Carlson and Jones, 1998). Live cells and the presence of the type III secretion system are required to activate this system (Zierler and Galán, 1995). Sip A-D, InvJ and SpaO (Inv K), examples of type III virulence factor secretion system in *Salmonella* species mediate the invasion of non phagocytic cells by *Salmonella*, are similar in function to Yops in *Yersinia* species (Finlay and Falkow, 1997).

Internalized bacteria may enter deeper tissues and also survive and replicate (Betts and Finlay, 1992). Other factors including a virulence plasmid are responsible for survival and growth of the bacteria within the host lymphatic system (Collazo and Galán, 1997) and also development of the disease symptoms (Salyers and Whitt, 2002a). Once internalized, *Salmonella* resides in large vacuoles and multiplication occurs after 4 hours of lag time (Finlay, 1994; Finlay and Falkow, 1997) inducing an inflammatory response (Gray and Fedorka-Cray, 2002). Studies of *Salmonella* invasion have been done mostly with *S.* Typhimurium, which causes a systemic disease in mice that is similar to typhoid fever in humans (Brumell *et al.*, 1999). Figure 2.1 shows a schematic diagram of *Salmonella* invasion of epithelial cells.

2.3 Methods for study of bacteria – host interactions

Selecting a suitable experimental system to investigate the interaction of the bacteria and host cells is a crucial decision. If an imperfect system is chosen, the results of the experiment will not be reliable, even if the procedure is performed accurately. There are different systems available for studying bacteria-host cell interactions and each system has it own advantages and disadvantages.

2.3.1 Human subjects

Ideally, using humans is the best and most accurate way of investigating the interaction between bacteria and the human body. But, it is not always feasible to use human volunteers. Even if safety and ethics were not the issue, the cost of such experiments will be a limiting factor (Salyers and Whitt, 2002b).

At least 3 phases of clinical trials with human subjects are required to get FDA approval for a new drug. Usually human subjects are used only in the final stage of testing in clinical evaluation of therapeutic agents (Ansel, Popovich, and Allen, 1995).

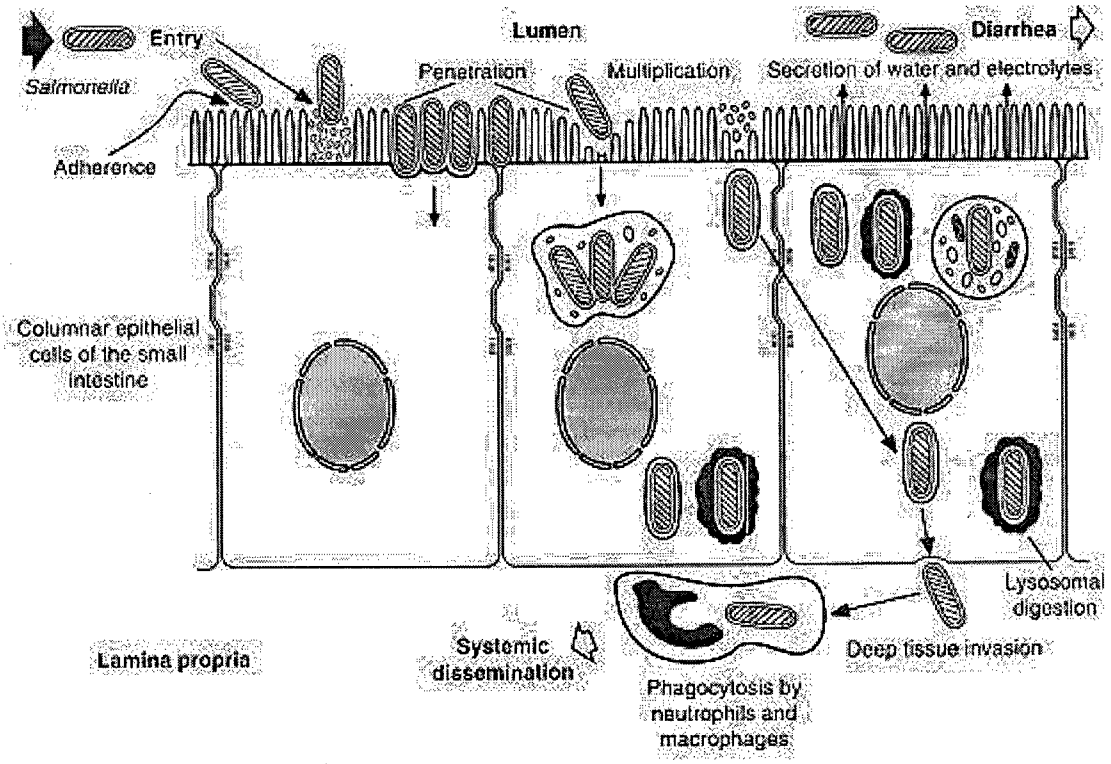

Figure 2.1: Schematic diagram of *Salmonella* invasion of epithelial cells. (Reprinted with permission from the *Salmonella* in Microbook edited by Samuel Baron, University of Texas)
http://www.pitt.edu/~super1/lecture/cdc0081/004.htm

2.3.2 Animal studies

The problems associated with the use of human volunteers to test bacteria host cell interaction are obvious and a need for practical substitutes has led to the use of other means. Animal models are usually good alternatives for human subjects for investigating bacterial infections. Laboratory animals can be kept in controlled environments in terms of diet and living conditions. Consequently, more consistent and reproducible results are expected compared to human subjects. Animals that are mostly used in these types of experiments are mice and guinea pigs (Salyers and Whitt, 2002b). Guinea pigs have been used to study adherence of enterotoxigenic *Escherichia coli* (Ashkenazi and Mirelman, 1987). The Sereny test is one of the tools employed for studying bacterial invasion using animal models. In this procedure the ability of the bacteria to cause keratoconjunctivitis in the cornea of test animals is examined and is related to the invasion of the cornea by the bacteria. The test is qualitative (Formal *et al.*, 1983).

Use of murine subjects is quite common in studying salmonellosis, especially for typhoid fever. Although no animal, other than humans, contracts true typhoid fever, once infected with *S.* Typhimurium, mice develop disease symptoms similar to human typhoid fever (Ziprin and Hume, 2001).

Although there are numerous advantages associated with using animal models, they have some drawbacks as well. Animals present a complex system and not all the factors can be controlled. It is often impossible to know the state of an animal's cells until the end of the experiment and this requires autopsy (Adams,

1990). When using laboratory animals, other issues such as high cost, inconvenience, low productivity, variability and ethical aspects should be taken into consideration.

Many bacteria that are pathogenic for humans cannot infect laboratory animals or do not produce the same symptoms. Sometimes, when ingestion of the bacteria does not induce a pathogenic effect, intraperitoneal injection of the bacteria may cause the infection. The disadvantage of this method is that, since bacteria will by-pass the intestinal colonization and invasion stages, the results will not be useful for investigating the invasion process (Salyers and Whitt, 1994).

2.3.3 Cell culture

In spite of all the advantages of using animal models a simpler and more easily controlled alternative model is preferred. Mammalian, cultured cell systems have been developed to substitute for animal models in studying bacteria-host interactions. Cell cultures are suitable models for investigating the bacteria-host interaction. They are more cost efficient and controllable than animal subjects (Betts and Finlay, 1992). They can be monitored by microscopy on a regular basis. It is essential to use healthy cells in the experiment. Cell culture provides the opportunity for quantitative estimation of the proportion of cells that are viable. Moreover their simplicity and consistency result in more reproducible outcomes than human subjects and animal models. Cell cultures provide a homogeneous population of cells, with identical genetic composition, which are growing in a controlled environment (Adams, 1990).

The recent major advances in understanding the pathogenesis of different pathogens are partly indebted to development of cell culture methods, which provide a rapid tool for such research.

Despite all the mentioned advantages, cell cultures also have limitations, which should be kept in mind especially when interpreting the results. In order to design a more reliable experiment, these limitations should be thoroughly understood. Since cells can undergo mutation during the continuous passaging, cell lines from the same source, and different passage number may not necessarily be identical (Salyers and Whitt, 2002b). It is important to keep a record of passage numbers and work within certain cell passages.

Numerous cell lines, including Caco-2, HEp-2, Henle and HeLa, have been used to study bacterial-host interactions (Carbonare, Silva, Trabulsi and Carneiro-Sampaio, 1995). Different quantification methods have been used to study the adhesion/invasion of bacteria to host cells. These methods include, light and electron microscopy, Enzyme Linked Immunosorbent Assay (ELISA), bacteria radiolabelling, and flow-cytometry (Alwan *et al.*, 1998). The gentamicin survival assay has become a standard technique to study bacterial invasion of mammalian cells (Tang, Foubister, Pucciarelli, and Finlay, 1993).

2.3.3.1 HeLa cells

HeLa cell lines, obtained from human cervix carcinoma, are one of the most extensively used cell monolayers for study of various bacterial invasion procedures

(Terebiznik Mauricio *et al.*, 2002; Roof, Kramer, and Roth, 1992). HeLa cells do not possess phagocytic property (Goluszko, Popov, Selvarangan, Nowicki, Pham, and Nowicki, 1997). Moreover, HeLa cells make good models for the study of pathogenicity because of their flat structure and cytoplasmic area (Francis and Thomas, 1996) which facilitates examination with a microscope. HeLa cells have been specifically used in studying *Salmonella* invasion (Mills and Finlay, 1994), virulence genes (Pfeifer, Marcus, Steele-Mortimer, Knodler, and Finlay, 1999).

2.4 Natural anti-bacterial factors

The increase in global demand for natural products has opened a new avenue of research. To satisfy this demand, food researchers are searching for natural substitutes to replace chemical additives used in food production. Safety issues and the emergence of multidrug resistant bacteria have become areas of concern pertaining to use of antibiotics and food preservatives. Naturally ocurring antimicrobials are abundant in the environment. These compounds can be found in animals and plants and they are employed as host defense mechanisms (Naidu, 2000a). There is currently interest in extraction and utilization of biologically active substances from their natural source. There is speculation that these compounds may be used in functional food formulations as natural bioactive ingredients or may be used as additives for other food systems (Facon, Skura and Nakai, 1993).

The natural antimicrobial agents have been thoroughly reviewed by Naidu (2000a). In the following section of this literature review, the focus will be on antimicrobials derived from milk or so-called "lacto-antimicrobials".

2.5 Natural antimicrobials in milk

Epidemiological studies revealed that breast-fed infants are more resistant to intestinal and respiratory infections (Kovar, Serdula, Marks, and Fraser, 1984). One of the earliest immunological observations was the apparent presence of factors in the early lactation of mammary secretions of certain species that prevented diseases. Colostrum, a mammary secretion during the first 72-96 hours after parturition (Scammell, 2001), is a complex fluid that provides nutrition requirements as well as biologically active compounds for the newborn mammal. Bovine colostrum has already been used to prepare a commercial product called "immune milk" that is rich in immunoglobulins, for patients suffering from gastrointestinal infections (Pakkanen and Aalto, 1997).

Ancient physicians used bovine colostrum to treat various medical problems. Long before the widespread use of antibiotics and anti-inflammatory drugs, medical doctors used colostrum to fight pathogens and also treat rheumatoid arthritis (Rona, 1998). In two independant *in vivo* studies, colostrum preparations containing antibodies against rotaviruses successfully protected infants who were admitted to hospital, from diarrhea (Davidson *et al.*, 1989; Ebina *et al.*, 1985).

Human colostrum and milk inhibit the adhesion of enteropathogenic *Escherichia coli* (EPEC) and the invasion of cultured cells by enteroinvasive *Escherichia coli* (Carbonare *et al.*, 1995; Schroten *et al.*, 1992). Araujo and Giugliano (2000) also reported that human milk and its fractions have inhibitory activity against adherence of diffusely adherent *Escherichia coli* and enteroaggregative *Escherichia coli* (EAEC).

Different components of milk such as immunoglobulins, lysozyme, lactoferrin, lactoperoxidase and more recently glycolipids, have been tested for their anti-bacterial activity (Schroten *et al.*, 1992).

2.5.1 Milk protein antimicrobials

The immunologically important bioactive agents in milk can be classified as specific and non-specific factors. Although some oligosaccharides and glycolipids (Sprong, Hulstein and van der Meer, 2002) have also been reported to have immunological properties, the reported bioactive compounds have mostly been proteins and peptides (Ekstrand, 1989). Some of these proteins are listed in Table 2.1.

Table: 2.1. Concentration of anti-microbial proteins in bovine milk and colostrum

Protein	**Concentration (mg mL^{-1})**	
	Milk	**Colostrum**
Immunoglobulin G	0.31-0.40[a]	52-87[a]
Lactoferrin	0.1[a]	1.5-5[a]
Lactoperoxidase	0.01-0.03[b]	-
β–Lactoglobulin	3.2[d]	5[c]
α–Lactalbumin	1.2[d]	-

[a](Pakkanen and Aalto, 1997), [b](Naidu, 2000c),[c] (Shah, 2000),[d](Grappin, 1992)

2.5.1.1 Immunoglobulins

In an attempt to find the most active anti-bacterial component in milk, many milk proteins and peptides have been studied for their anti-bacterial activity, amongst which immunoglobulins, especially type G (IgG), have received the most attention. Bovine neonates rely on the immunity, which is transferred from milk during the first few days postpartum. IgG is the most prevalent immunoglobulin in bovine milk. The concentration of IgG is milk and colosturm is presented in Table 2.1, although the concentration range may be greater than the values reported by Pakkanen and Aalto (1997) based on different physiological and environmental factors (Pritchett, Gay, and Besser, 1991). IgGs are comprised of two identical light chains and two identical heavy chains with molecular weights of around 25 kD and 50 kD respectively. The chains are joined by disulphide bonds, which can be dissociated by sulphydryl reducing agents. Papain digestion of IgG breaks the molecule into two upper portions (Fab) and one lower portion (Fc) (Larson, 1992). Figure 2.2 shows the schematic picture of IgG and the position of the disulphide bonds.

Protein G, obtained from the group G streptococci, efficiently binds to the constant region (Fc) of all subclasses of immunoglobulins (Guss *et al.*, 1986). This phenomenon has been applied to development of special affinity chromatography columns for purification of immunoglobulin G.

Bovine milk immunoglobulins and their role in providing passive immunity have been studied extensively. Some of the bioactivities attributed to IgG are: activation of complement-mediated bacteriolysis; opsonisation; inhibiting bacterial

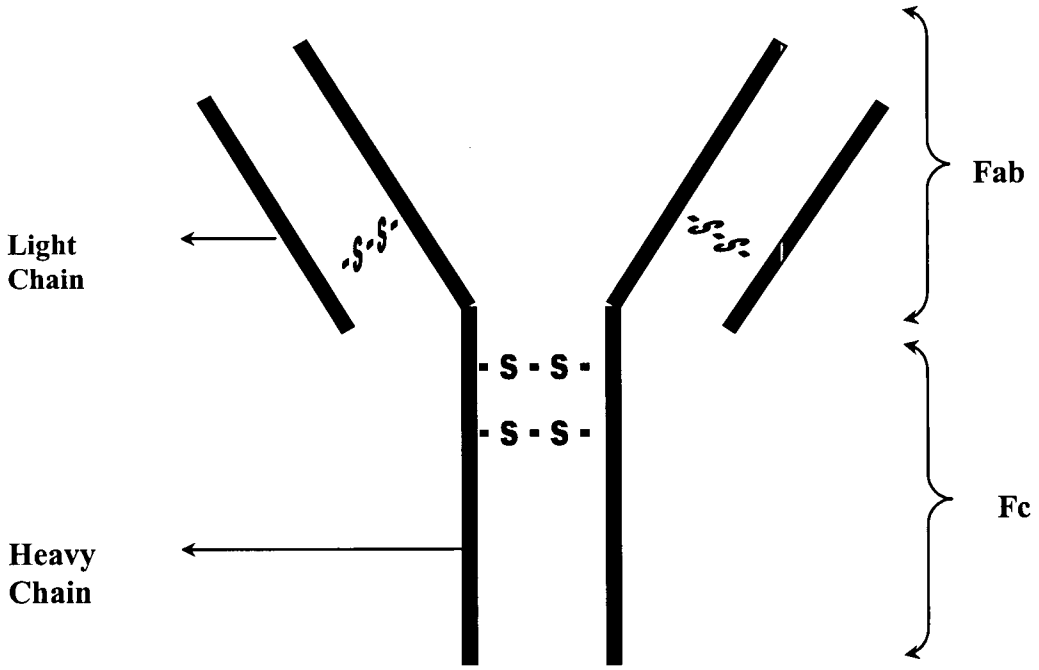

Figure 2.2. Schematic picture of immunoglobulin G, showing two heavy chains and two light chains joined by disulphide bonds. (Adapted and simplified from Larson, 1992).

adhesion; bacteria aggregation; toxin and virus neutralisation (Korhonen, Marnila and Gill, 2000).

The idea of inclusion of bovine immunoglobulins in food preparations was conceived as early as the 1950s. Since then, the idea has grown and the number of studies on food formulations using bovine immunoglobulin has increased (Korhonen, 2000). Fortification of infant formula with immunoglobulins to provide passive immunity against pathogens has been recommended by several researchers (Nakai, 1991; Korhonen, 2000; Facon *et al.*, 1993). Raising antibodies against specific pathogens in cows and subsequent isolation and incorporation into food, feed and pharmaceutical formulations has reached the industrial level (van Hooijdonk, Kussendrager and Steijns, 2000). Immune milk preparations, containing specific antibodies against a variety of pathogens, have been successfully used in treating patients suffering from infectious disease. Production costs and maintaining the immune activity during and after ingestion still are factors limiting wide usage of these preparations (Pakkanen and Aalto, 1997). The downside of the utilization of immunoglobulins in food formulations is that oral administration of immunoglobulin preparations derived from bovine milk and chicken eggs have been reported to cause allergic reactions in milk and egg-sensitized individuals. This may limit their usage in immune preparations (Bernhisel-Broadbent, Yolken and Sampson, 1991). Also there have been some issues raised regarding the role of bovine IgGs in inhibition of antibody secretion (Kulczycki, Nash, Bertovich, Burack, and MacDermott, 1987; Nash *et al.*, 1990).

2.5.1.2 Lactoferrin

Lactoferrin and its well-known pepsin digest, lactoferricin, have been extensively studied for their anti-bacterial properties. Lactoferrin is a cationic metal-binding glycoprotein, composed of 703 amino acids (Levay and Viljoen, 1995) and has a molecular weight of 80 kDa. It can be found in milk and other body fluids as well as various exocrine secretions (Facon, 1995). The concentration of lactoferrin in human colostrum and milk are reported to be 6.7 and 2.6 mg mL^{-1} respectively (Naidu, 2000b).

Lactoferrin has been widely studied for its iron absorption properties and consequent protection against enteric infections. Once infection occurs, neutrophils engulf the microorganism and secrete lactoferrin and through providing a hypoferraemic situation the growth of the microorganisms will be impeded (van Hooijdonk *et al.*, 2000). This conclusion is drawn from research showing the disappearance of anti-bacterial activity when iron saturated lactoferrin was used instead of lactoferrin. Recent research suggests that there are other mechanisms by which lactoferrin could elicit its antimicrobial activity (Naidu, 2000b).

Studies show that lactoferrin is a multifunctional bioactive molecule with a critical role in many important physiological pathways. It has been shown that lactoferrin can confer its anti-bacterial activity when administrated orally, intravenously and also intraperitoneally. Different mechanisms are suggested for each type of administration (van Hooijdonk *et al.*, 2000). Lactoferrin could elicit a variety of inhibitory effects including stasis, cidal, adhesion-blockade, cationic, synergistic or

opsonic effect against a broad-spectrum of microorganisms (Pakkanen and Aalto, 1997; Naidu, 2000b). Lactoferrin was also reported to possess other properties such as anti-inflammatory and immunomodulatory activity. The mechanism of action is not clearly understood (Naidu 2000b; van Hooijdonk *et al.*, 2000). Interestingly, it has been also reported that the active peptides of hydrolysed lactoferrin (lactoferricin) are more effective in conferring anti-bacterial activity than lactoferrin itself (Facon and Skura, 1996; Branen and Davidson, 2000).

For two decades lactoferrin has been proposed as a food additive, either as a preservative or for the prevention of gastroenteritis (Tomita, 1994) and has already been applied as an active ingredient in infant formulae and health foods in South-East Asian countries. It is also used as a therapeutic and prophylactic agent to control intestinal illnesses and mucosal infections. There have been over 100 patents filed on this molecule in the past decade. Undoubtedly it is emerging as one of the leading natural microbial blocking agents in food safety and preservation (Naidu, 2000b)

Current annual commercial production of lactoferrin from bovine milk is 100 metric tons and this figure is continuously increasing (Naidu, 2000b). Lactoferrin is also reported to have the ability to interact with surfaces and other molecules (Naidu and Arnold, 1997; van Hooijdonk *et al.*, 2000). A receptor mediated binding activity between lactoferrin and some macromolecules has been found. Lactoferrin receptors have been identified on intestinal cells, monocytes, macrophages, neutrophils, albumin, β-lactoglobulin, lysozyme and bacteria (Levay and Viljoen, 1995). The role

of these receptors in the functional activity of lactoferrin has not been fully investigated.

Recently, another bioactive property of lactoferrin has been identified and there is a growing interest in characterizing its antagonistic activity against bacterial invasion and adhesion. Anti-invasive/anti-adhesive properties of lactoferrin on *Listeria monocytogenes* (Antonini *et al.*, 1997) and group A streptococci (Ajello, Greco, Giansanti, Massucci, Antonini and Valenti, 2002) have been reported.

2.5.1.3 Lactoperoxidase

Lactoperoxidase, an oxidoreductase secreted into milk, plays an important role in protecting the lactating mammary gland and the intestinal tracts of the newborn infants against pathogenic microorganisms. As cited by Korhonen (1980), Hanssen, in 1924, discovered the bactericidal effect of fresh milk, which he attributed to the oxidizing enzyme present in milk. The antimicrobial activity of lactoperoxidase was first noticed in an incidence of improper growth in cheese starter cultures (Ekstrand, 1989).

Lactoperoxidase has a molecular weight of 78 kDa and has 612 amino acids (Kussendrager and van Hooijdonk, 2000). In order to benefit from the anti-bacterial activity of lactoperoxidase, it should be used in combination with hydrogen peroxide and thiocyanate (SCN^-). The combination is called the lactoperoxidase system (van Hooijdonk *et al.*, 2000). Lactoperoxidase catalyses the oxidation of thiocyanate and iodide ions and generates highly reactive oxidizing agents, which demonstrate a broad

spectrum of antimicrobial effects against bacteria, fungi and viruses (Pakkanen and Aalto, 1997; Naidu, 2000c). Kussendrager and van Hooijdonk (2000) reported a 30% reduction in mortality rate of rainbow trout receiving a diet containing lactoperoxidase system and also a 58% reduction in sea lice infection in Atlantic salmon using lactoperoxidase system in the growth tank. The proposed mechanism by which these oxidizing agents confer the anti-bacterial activity is through oxidizing the sulphydryl groups in bacterial proteins and damaging the outer membrane (Pakkanen and Aalto, 1997; van Hooijdonk *et al.*, 2000).

The lactoperoxidase system has found use in a variety of applications as a commercial natural preservative. The antimicrobial activity of lactoperoxidase system has been used to preserve raw milk in warm climates, through the addition of hydrogen peroxide and thiocyanate (Naidu, 2000c; van Hooijdonk *et al.*, 2000). Also it has been recommended for use as a preservative and antimicrobial agent in cosmetics, mouthwashes, toothpaste and chewing gums (Kussendrager and van Hooijdonk, 2000; van Hooijdonk *et al.*, 2000). A synergistic interaction in inhibition of diarrhea was noticed in an *in vivo* study in calves when the lactoperoxidase system was used with lactoferrin (Van Leeuwen, Huisman, Kerkhof, and Kussendrager, 1998).

2.5.1.4 β-Lactoglobulin

β–Lactoglobulin, the major whey protein in bovine milk, has a molecular weight of 18 kDa in monomer and 36 kDa in dimer form. Palmer isolated β-

lactoglobulin in 1934 for the first time (Sawyers, Kontopidis, and Wu, 1999). Although β-lactoglobulin has been studied extensively, its biological function and importance are not fully known. *In vitro* studies have shown that β-lactoglobulin can bind various ligands, mostly small hydrophobic molecules. Several biological functions have been attributed to β-lactoglobulin, such as binding retinol (Fox and Flynn, 1992) and fatty acids (Perez and Calvo, 1995; Dufour, Genot and Haertlé, 1994). Binding activity between β-lactoglobulin and α-lactalbumin has also been reported (Hambling, McAlpine and Sawyer, 1992). As a result of environmental conditions such as pH, temperature, concentration and presence of other substances, β-lactoglobulin undergoes different conformational changes, which in turn affect its binding activity. Sedimentation studies revealed that bovine β-lactoglobulin is a dimer under different physiological conditions, for instance at neutral pH (Dufour *et al.*, 1994). The quantity of β-lactoglobulin in bovine milk, which varies with season and time since parturition, can range from 1.8 to 5 mg mL^{-1}. Human milk does not contain β-lactoglobulin (Shah, 2000). Two main genetic variants, A and B, have been isolated from bovine β-lactoglobulin and are the ones most often found in commercial preparations. Different breeds of cows may have different variants. For instance variant C can only be found in Jersey cow milk. Genetic variants, which have been identified and sequenced so far from bovine milk are types A, B, C, D, E, F, G and Dr (Hambling *et al.*, 1992).

Recently, antimicrobial activity has been attributed to β-lactoglobulin. In one study, researchers demonstrated that β-lactoglobulin can successfully inhibit the

fimbrial adhesion of *Escherichia coli* and *Klebsiella oxytoca* to human ileostomy glycoproteins suggesting that β-lactoglobulin may have some antimicrobial activity (Ouwehand, Salminen, Skurnik, and Conway, 1997). Four bactericidal domains, isolated and characterized from bovine β-lactoglobulin, were negatively charged and were effective against gram-positive bacteria (Pellegrini, Dettling, Thomas, and Hunziker, 2001).

2.5.1.5 α- Lactalbumin

α-Lactalbumin, with 14 kDa molecular weight, is the second major whey protein in bovine milk and the dominant whey protein in human milk. It has calcium binding property (Hiraoka, Segawa, Kuwajima, Sugai, and Murai, 1980) and does not coagulate upon heating. This phenomenon is concentration dependant. It has a close homology with chicken egg lysozyme (Brew, and Grobler, 1992). The most significant biological function attributed to α-lactalbumin is its role in lactose biosynthesis (Hambraeus, 1992). In the search for natural antimicrobials in milk, α-lactalbumin also has been tested for its inhibitory activity against different microorganisms. The folding variant of the α-lactalbumin from human milk showed strong bactericidal activity against *Streptococcus pneumoniae*, while native α-lactalbumin had no effect (Håkansson *et al.*, 2000). Enzymatic digestion of bovine α-lactalbumin produced three polypeptides with bactericidal activity (Pellegrini, Thomas, Bramaz, Hunziker, 1999).

2.6 Nutraceuticals and functional foods

Although the terms nutraceutical and functional foods are sometimes used interchangeably, according to Health Canada's definition they are referred to as two different categories of products. "A *nutraceutical* is a product isolated or purified from foods that is generally sold in medicinal forms not usually associated with food. A nutraceutical is demonstrated to have a physiological benefit or provide protection against chronic disease". "A *functional food* is similar in appearance to, or may be, a conventional food, is consumed as part of a usual diet, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions" (Health Canada, 2002).

In this thesis the term functional foods is used to refer to foods that can deliver more than hunger satisfaction or basic need components to the human body. Functional foods are designed to promote certain physiological function (van Hooijdonk *et al.*, 2000). Given the fact that the immunological properties of colostrum have been well studied and acknowledged, several milk products containing colostrum are commercially available and are being marketed throughout Europe and United States. The target market for these products is individuals with immunodeficiency, nutrition deficiency and athletes (Uruakpa, Ismond, Akobundu, 2002). Incorporating bioactive compounds of food origin into new food products has become one of the predominant areas of food formulation research (Meisel, 2001).

2.7 Optimization methods

When formulating products, there is a need to find the optimum levels of each component to satisfy the objective of the formulation. The dependent variable or response, which should be optimized, is selected based on the objective of the design. In an ideal situation, to achieve the best formulation, all variables should be varied concurrently and their effect on the response must be studied. Obviously, this approach especially when there are several variables, is extremely difficult if not impossible to achieve. Using mathematical models in formulation of food products minimizes the cost by reducing the number of required experiments (Arteaga, Li-Chan, Vazquez-Arteaga, Nakai, 1994). A number of experimental designs have been applied in food product formulation. Experimental design dates back to 1920 when Sir Ronald Fisher applied statistical design to agricultural research (Anonymous, 1990). Since then, new techniques have emerged. Today, there are many techniques, which can be used for formula optimization. In this thesis Random Centroid Optimization was used and is presented in more detail below.

2.7.1 Random Centroid Optimization (RCO)

Random Centroid Optimization program is a multifactor program developed at the University of British Columbia and it is a useful tool for finding the optimum level of components in formulations. The program is versatile and has been shown to handle optimizing up to 20 factors (Dou, Toma and Nakai, 1993) and theoretically it can even optimize more than 20 factors. This method belongs to the sequential

methods category of optimization techniques (Nakai, 1990) and works fine for the formulations in which the relation between the components and working equations are not known and experimentation is required for optimization (Nakai, Dou, Lo, and Scaman, 1998). RCO technique has the advantage of finding the global optimum with a relatively small number of experiments (Dou *et al.*, 1993; Nakai *et al.*, 1998).

The program has been designed by combination of random search, centroid search and mapping features. Briefly, in this program, the number of factors, name of the factor and range for each factor should be defined for the program. With the aid of a regulated random generator, different combinations are designed with varying levels of factors (Dou *et al.*, 1993). The regulated random generator has been incorporated to increase the efficiency of the program in finding the global optimum and to avoid misleading results. The program divides the search space into 5 zones, identified as level values. In each zone, comprising 20% of the search range, at least one level value for each factor will be randomly chosen. Also the average of all "level values" should be in a specific percentage range from the midpoint of the search space based on the number of factors. This regulation gives the values the better chance of uniform distribution in the search space and prevents accidentally homing in on values in a narrow range (Nakai, 1990). The formulations designed by the random generator will be subjected to experimentation and the results will be used as responses in centroid search. Centroid generates a few more combinations with a narrower range around the best points picked from the random search responses. Again the combinations will be subjected to experimentation. A response surface will be developed, based on all responses, by a mapping function, which helps with

visualizing the trend or the area where the global optimum may reside. In the mapping section, the search space is divided into three equal zones with the data points spread all over the search space.

The trend lines in each zone are drawn between data points in the same subdivision. The trend lines point to the location where the probability of the global optimum residing is higher. The program has the capability of eliminating irrelevant factor(s), which reduces the number of further experiments. In the mapping section, use of the "locate optimum function" (Locate Optm) of the program automatically gives the optimum based on the best responses received. A manual shifting function, as a scroll box underneath each map, is provided for the operator to change the optimum point and map the results based on the new optimum. This option gives the operator freedom of interpreting the results of mapping based on the maps with increased trend lines, which highly affects the efficiency of the interpretation.

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CHAPTER THREE

Anti-invasive activity of bovine colostrum, immunoglobulin G-free fraction, lactoferrin, α-lactalbumin, β-lactoglobulin and lactoperoxidase on S. Typhimurium invasion of HeLa cells

3.1 Introduction

Naturally occurring antimicrobial factors in foods are receiving renewed interest. Part of this interest comes from the fact that food manufacturers are interested in incorporating these factors in food formulations, especially infant food preparations, as natural antimicrobial agents to satisfy the expectations of the new age, health conscious consumers (Naidu, 2000). Another concern is the emergence of antibiotic resistant bacteria and the consequent complications which, shifts food consumption trends towards utilization of more natural products (Naidu, 2000).

A brief review of the relevant databases revealed an astonishing number of research papers dedicated to discovering new anti-bacterial agents from natural sources. More than 500 natural anti-bacterial peptides have been characterized during the past two decades of research (Pellegrini, Dettling, Thomas and Hunziker, 2001). The anti-bacterial effect of bovine and human milk and colostrum has been well documented (van Hooijdonk, Kussendrager and Steijns, 2000). Long before the discovery of antibiotics, milk proteins were used as a non-specific treatment of infections. There is a growing recognition of milk proteins and peptides as potential stimulators of host defence against infections and the possibility of medical application (Lahov and Regelson, 1996).

Most research on antimicrobial activity of the milk components focused on changes in colony counts after introducing the test sample to a microbial culture. As discussed elsewhere in this thesis, such methods do not show the bacteria-host cell interactions. Cultured eukaryotic cells are becoming an important tool in studying

bacteria-host interactions since the cells provide reproducible and less expensive models than animal models. Cultured eukaryotic cell models have been used especially in studying bacterial adhesion and invasion. Bacterial adhesion followed by invasion is the first step of creating disease by some pathogens, such as *S.* Typhimurium. The development of disease caused by bacteria can be prevented by inhibiting the adhesion and invasion phenomena (Carlson and Jones, 1998).

Human milk and its components have been studied comprehensively and it is evident that there are some anti-bacterial agents in human milk. Human milk anti-bacterial glycoconjugates have been extensively reviewed (Newburg, 1999). A recent review of databases did not reveal a broad range of research on the anti-invasive activity of non-IgG fraction of bovine milk. Whey proteins are also receiving attention as these valuable proteins are wasted in large amounts as a by–product of cheese processing (Jelen and Lutz, 1998). Their wastage not only deprives us of a very valuable protein source but also causes environmental problems. There are numerous methods whereby whey can be utilized for animal feed, farmland irrigation, lactose and lactose derivative production (Morr, 1992). Whey also can be dehydrated or concentrated and used in food formulations in confectionary products, beverages, frozen desserts (Morr, 1992) and also research studies are underway to isolate components which can be used as ingredients in health food formulations and functional foods (Decker, Tak, Verdecchia and Horgan, 1997; Bounous, 1997; Smithers *et al.*, 1997; Perraudin and Reiter, 1997). Whey proteins, mainly lactoferrin and lactoperoxidase, have received a great deal of attention for their reported anti-bacterial activity.

The purpose of the present study was to evaluate the anti-invasive activity of bovine colostrum, the immunoglobulin-free fraction of bovine colostrum and whey proteins using HeLa cell culture as a model system and *S.* Typhimurium as the challenge bacteria.

3.2 Materials and Methods

3.2.1 Buffers and reagents:

3.2.1.1 Protein G chromatography buffers

Binding buffer: (Acetate buffer pH 5): Mixture of 0.2M sodium acetate buffer and 0.2M acetic acid (70:30 v/v).

Elution buffer: 0.05M glycine-HCl buffer pH 2.8: 0.2 M glycine and 0.2N HCl in the ratio of 25% : 8.4% in distilled water.

3.2.1.2 SDS-PAGE

30% Acrylamide: 0.8% bisacrylamide: Acrylamide 30 g, bisacrylamide 0.8 g in 100 mL distilled water.

Lower stock (1.5 M Tris, pH 8.8): Tris base 18.165 g, SDS 0.4 g in 100mL distilled water; pH adjusted with HCl.

Upper stock (0.5 M Tris, pH 6.8): Tris base 6.055 g, SDS 0.4 g in 100 mL distilled water; pH adjusted with HCl.

Running buffer 10X (0.25M Tris, 1.92 M glycine): Tris base 15.14 g, glycine 72.05 g, SDS 5.0 g in 500 mL distilled water (pH of single strength solution was 8.3).

Sample buffer: Upper stock 1.0 mL; SDS 0.1 g; glycerol 3 mL; β-mercaptoethanol 0.2 mL; bromophenol blue 10 mg in 10 ml distilled water. Aliquots were dispensed in microtubes and kept frozen.

3.2.1.3 ELISA

Phosphate buffered saline (PBS) pH 7.4: Mix 8.0 g NaCl; 0.2 g KH_2PO_4; 1.15 g Na_2HPO_4; 0.2 g KCl and 0.2 g NaN_3 in 1 L distilled water.

Carbonate coating buffer pH 9.6: 1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 0.2 g NaN_3 in 1 L distilled water.

PBS-Tween: Tween 20; 0.5 mL in 1 L PBS

Blocking buffer: 0.5 g Skim milk powder (Carnation, Nestle, Don Mills, ON Canada) reconstituted in 100 mL PBS and boiled for 10 minutes in a beaker with a lid to prevent the volume change due to vaporization.

Diethanolamine buffer pH 9.8: 97.0 mL diethanolamine, 0.1 g $MgCl_2.6H_2O$; 0.2 g NaN_3, diluted in distilled water to 1 L, pH adjusted to 9.8

3.2.1.4 Biuret protein assay

Biuret reagent: 2.25 g Sodium potassium tartrate; 0.75 g $CuSO_4.5H_2O$; 1.25 g KI, all dissolved in order in 100 mL 0.2 M NaOH and made up to 250 mL with distilled water.

3.2.1.5 Cell culture

PBS^{--}: the same recipe as PBS in ELISA except that no NaN_3 was added, PBS^{--} was sterilized at 121° C and 15 psi for 20 min.

PBS^{++}: 0.1 g $MgCl_2.6H_2O$; 0.1 g $CaCl_2.6H_2O$ in 1 L PBS^{--} (filter sterilized)

3.2.2 Samples

3.2.2.1 Colostrum

In immuno-supplementation studies, bovine colostrum is often used as a source of immune compound, since the concentrations of active compounds are higher than in milk. Bovine colostrum was used as a model in this study. Colostrum samples were obtained throughout the year, from the UBC Dairy Research Center, Agassiz, British Columbia, Canada, during the first 24 hours postpartum. Samples were collected in polyethylene containers and transported frozen to the University of British Columbia and kept at –15°C until required.

3.2.2.2 Whey proteins

Bovine lactoferrin (Sigma 4765), α-lactalbumin type III (Sigma L6010), β-lactoglobulin (mixed variant) 3X crystallized (Sigma L0130), β-Lactoglobulin A (Sigma L7880), β-lactoglobulin B (Sigma L8005) and lactoperoxidase A_{412}/A_{280} 0.7-0.85 (Sigma L2005) were used in this study. Whey proteins were weighed using microbalance (Mettler ME 30, Mettler Inc. Switzerland). Purity of the proteins was verified by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gel concentration was 12.5% and the gels were stained with 0.1% solution of Coomassie blue. Whey protein concentrations were assessed by UV absorbance at 280 nm of a 1 % solution through a path length of 1 cm using the extinction coefficients of lactoferrin 15.1, α-lactalbumin 20.9 and β–lactoglobulin 9.5 (Sober, 1970).

3.2.2.3 Preparation of skim colostrum

Frozen samples were thawed at 4°C. From each of 12 colostrum samples, 50 mL was taken, pooled and used in this study. Since the presence of fat would interfere with the proceeding steps, all samples were skimmed by centrifugation in 200 mL stainless steel cannisters at 16,600 × g (Sorvall® RC 5B plus, Sorvall Instruments, Dupont, New Town, CT, USA) for 20 minutes at 4°C (Facon, 1995). Solidified fat was removed from the supernatant surface of each centrifuge cannister. Colostrum was filtered through cheese-cloth to exclude any remaining solidified fat residues. The skimmed colostrum samples were kept frozen at -15°C until required.

3.2.2.4 Colostrum whey preparation

Colostral whey was prepared by acid precipitation of casein. Skimmed colostrum was diluted 1:1 with de-ionized distilled water and acidified with 1N HCl to a final pH of 4.6. Samples were incubated in a water bath at 40°C for 30 minutes to facilitate casein precipitation. The acid-precipitated casein was removed by filtration through cheese cloth. The filtrate was the final material for the study.

3.2.2.5 Freeze drying of sample

A 100 mL sample of skimmed colostral whey was freeze-dried (Labconco, Kansas City, MO. USA) at 80 μmHg and condenser at temperature of –50°C.

3.2.3 Separation of immunoglobulin G

Affinity chromatography was conducted to separate immunoglobulin G (IgG) from skimmed colostrum. Protein G–agarose ImmunoPure®, Immobilized Protein G, (Pierce Chemical Co. Catalogue number 20398) was packed in a BioRad plastic 10 mL column. The column containing 1.5 mL of immobilized protein G was equilibrated with 4 bed volumes of 0.2 M sodium acetate buffer pH 5.0. Freeze-dried skimmed colostral whey sample was reconstituted in 50 mL of distilled water. An aliquot of 500 μL was first diluted 1:1 with binding buffer and applied to the affinity column. Unbound fractions, eluted using acetate buffer at a flow rate of 0.27 mL min^{-1}, were collected in 2 mL volumes in tubes and absorbance of the fractions was

monitored with a UNICAM UV/VIS UV2 Spectrophotometer (ATI UNICAM) at 280 nm. Completion of washing was determined when effluent absorbance approached baseline. The bound fraction was eluted with 0.05M glycine-HCl, pH 2.8, at a flow rate of 1 mL min^{-1}. Elution, which was monitored by spectrophotometry at 280 nm to determine the completion of washing, was stopped when the absorbance approached baseline. Respective peak fractions of unbound fraction (P1) and bound fraction (P2) were pooled.

3.2.4 Determination of IgG by enzyme linked immunosorbent assay (ELISA)

Sandwich ELISA (Kummer, Kitts, Li-Chan, Losso, Skura and Nakai, 1992) was conducted on the pooled P1 fraction to ensure the complete separation of IgGs. Flat–bottomed 96 well polystyrene microtiter plates (Immulon® II, HB, Dynex Technologies, Inc. Chantilly, VA, USA, Catalogue number 3455) were coated with 100 µL of 1 µg mL^{-1} affinity isolated rabbit antibody to bovine IgG (Sigma, B-5645) in carbonate coating buffer and incubated for 60 minutes at 37°C. The wells were washed 5 times with PBS-Tween and 250 µL of blocking buffer was added to the wells. After 30 minutes of incubation the plates were washed 5 times with PBS-Tween and 100 µL of serial dilutions of colostrum and other test samples in PBS-Tween were dispensed into the wells. Bovine serum IgG (Sigma I-5506) was used as standard. Purity of commercial IgG was checked by SDS-PAGE and protein concentration was calculated by using an extinction coefficient of 14 $1\%^{-1}$ cm^{-1} (manufacturer's data) for the absorbance of 1% solution at 280 nm. Following 60 minutes incubation at 37°C, the plates were emptied

and washed 5 times with PBS-Tween and 100 μL of rabbit anti-bovine IgG alkaline phosphatase conjugate (Sigma A0705, 1: 41,000 dilution in PBS-Tween) were used as secondary antigen. The plates were incubated for 60 minutes at 37°C and washed 5 times with PBS-Tween and once with distilled water. Then 100 μL of substrate (*p*-nitrophenyl phosphate, Sigma 104) solution in diethanolamine buffer were added to each well. Substrate solution contained 0.5 mg alkaline *p*-nitrophenyl phosphate per mL of diethanolamine buffer. Yellow colour development produced by the enzyme-substrate reaction was quantified at 405 nm using a microplate reader (Labsystems iEMS Reader MF, Labsystems Oy, Finland). Samples were assayed in quadruplicate.

3.2.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to monitor the separation of IgG from colostrum whey after passage through the protein G-agarose affinity column. SDS-PAGE buffers and the stacking and resolving gels were prepared according to Laemmli (1970). The gel concentration was 12.5%. Wide range molecular weight marker (Sigma, M 4038) and pure bovine IgG (Sigma I 5506) were used as standard. Samples were prepared in sample buffer with protein concentrations adjusted to 5-10 μg in 20 μL and run under reducing conditions at 75 volts, until the samples penetrated the resolving gel and then at 150 volts until the tracking dye reached the gel front using Bio Rad Power Pac 300 Electrophoresis Power supply and Mini-PROTEAN® II Slab Cell (Bio-Rad Laboratories, Hercules, CA, USA) gel electrophoresis system. The gels were stained with a solution of 0.1% Coomassie blue. Gels were destained in a solution of 30%

methanol and 10% acetic acid, shrunk in 50% methanol and dried using a vacuum gel drying system (Gel Slab Drier GSD-4, Pharmacia Fine Chemicals, Sweden).

3.2.6 Protein determination

Protein concentration was determined by Biuret protein assay. The conventional procedure was modified for microassay. Aliquots of 20 μL of test samples were added to each well of the microtiter plate. Biuret reagent (180 μL) was added to each well. Using the shaker option of the microplate reader, the solutions in the plate were mixed for 1 minute and incubated at room temperature for 20 minutes. The absorbance at 550 nm was measured using a microplate reader (Labsystems iEMS Reader MF, Labsystems Oy, Finland). Determinations were done in duplicate.

3.2.7 *S.* Typhimurium culture

S. Typhimurium SL1344 was generously provided by Dr. B. Finlay, Biotechnology Laboratory, University of British Columbia. Frozen bacterial stock was prepared from the *S.* Typhimurium culture on the LB agar plate using standard freezing procedure. Briefly, a loopful of bacteria from the plate was grown in 2 mL of LB broth (Difco 0402-17-0) tubes overnight at 37°C. The following day the culture was set on ice for 30 minutes and 1.2 mL of cooled bacterial culture was mixed with 0.4 mL of sterile cold 80% glycerol to achieve a final glycerol concentration of 20%. The mixture was then left on ice for 30-60 minutes and aliquots of 1 mL were

dispensed in sterile 2 mL microtubes (Sarstedt 72.695) and kept at – 80°C until required.

3.2.7.1 Regenerating *S.* Typhimurium culture from frozen stock

To regenerate the frozen stock, a semi-thawed bacterial culture was streaked on LB Agar (Difco 244520) plates and incubated for 24 hours at 37°C. Plates were kept refrigerated for a maximum of 4 weeks and were subcultured regularly. It was decided to subculture the bacteria up to 4 times and then regenerate a fresh culture from frozen stock in order to maintain consistency. Repeated cultivation of bacteria for longer periods of time has been reported to affect the pili 1 formation (Horiuchi, Inagaki, Okamura, Nakaya, Yamamoto, 1992). Presence of pili 1 is crucial in conferring invasiveness to *S.* Typhimurium (Horiuchi *et al.*, 1992).

3.2.8 HeLa cell cultures

3.2.8.1 Preparing HeLa cells frozen stock

Human cervical cancer cells, HeLa (ATCC, CCL-2), were used as a model for epithelial cells to study the invasion activity of *S.* Typhimurium. Cells were kindly provided by Dr. B. Finlay, Biotechnology Laboratory, University of British Columbia. Frozen stock was prepared using the standard method described by ATCC (Sharon Ruschkowski, Biotechnology laboratory, UBC, personal communication, 1997).

Briefly, cell monolayers were grown to confluency in a polystyrene 250 mL vented cap tissue culture flask (Sarstedt 83.1813.302) containing antibiotic free Minimum Essential Media (MEM) (GIBCO BRL catalogue number 11095-080) supplemented with 10% (V/V) Fetal Bovine Serum (FBS) (GIBCO BRL catalogue number 16000-036). Confluent cells were detached from the flask surface using trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA tetra sodium, GIBCO BRL catalogue number 25300-047). Cells were flushed with 4 mL of trypsin-EDTA followed by immediate removal of 3 mL of trypsin-EDTA from the flask. Cell lift up, which began after several minutes, was monitored with an inverted microscope (WILD inverted microscope, Switzerland). In order to stop the enzymatic reaction of trypsin, the flask was then rinsed with 10 mL of MEM containing 10% FBS. Serum contains trypsin inhibitor α-1-anti-trypsin and stops the enzymatic reaction. Detached HeLa cells were transferred to 50 mL (115 x 28mm) sterile centrifuge tubes (Sarstedt 62.559.001) and centrifuged for 15 minutes at 250 x g at 4°C (IEC Centra–7R International Equipment Company).

Meanwhile, one tube with 2.5 mL of MEM and 2.5 mL of FBS (tube 1) and another tube (tube 2), with 1.5 mL of MEM, 2.5 mL of FBS and 1 mL filter sterilized (0.2 μm Acrodisc® syringe filters catalogue number 4454, PALL Gelman Ltd, Ann Arbor, MI USA) dimethylsulfoxide (DMSO, Sigma D4540) were prepared and placed on ice. After the cells were sedimented, the old medium was removed by careful suction and 5 mL from tube 1 (FBS + media only) were added. Cells were gently re-suspended and clumps were broken up by several aspirations using a 5 mL pipet. The tube was cooled on ice for 15 minutes. Then contents of tube 2 (one with DMSO)

were added to the cooled culture and cells were re-suspended gently and thoroughly and the tube was left on ice for another 15 minutes to allow permeation of DMSO into the HeLa cells. One mL aliquots of cell suspension were dispensed into 1 mL cryovials (Nalgene catalogue number 5000-0012, Nalge Company, NY. USA), which were placed in an insulated container and left at -20°C. After 4 hours the container was transferred to -80°C over night. Vials were placed in storage cannisters on the next day and immersed in liquid nitrogen (approx. –196 ° C) tank until required.

3.2.8.2 Regenerating HeLa cells from frozen stock and subculturing

To regenerate the HeLa cells from the frozen stock, vials were removed from the liquid nitrogen storage tank and placed in a 37°C water bath to thaw the contents (Sharon Ruschkowski, Biotechnology laboratory, UBC, personal communication, 1997). At the same time, MEM was warmed to 37°C in a water bath. Cell viability was checked using the trypan blue dye exclusion method (Martin, 1994) and counting the viable cells on Neubauer hemocytometer grids (Albert Sass, West Germany). Contents of vials were aseptically transferred to a 25 mL tissue culture flask and 4 mL of fresh pre-warmed antibiotic-free MEM supplemented with 10% FBS was added. Cultured cells were incubated at 37°C under a humidified atmosphere of 5% CO_2: 95% air to allow attachment. In order to lower the concentration of DMSO, cells were washed with pre-warmed sterile PBS^{--} and provided with fresh pre-warmed MEM after one day. The cultured cells were monitored daily with a microscope (WILD inverted microscope, Switzerland) for morphology changes and growth trends

towards confluency. Regular microbial control was conducted by plate counting to ensure the wholesomeness of the culture. The cells were sub-cultured regularly by washing the cells with pre-warmed PBS^{--} followed by harvesting by the trypsin detachment procedure. Briefly, 4 mL of trypsin-EDTA were flushed over the cell monolayer followed by immediate removal of 3 mL of added trypsin-EDTA from the flask. Complete cell detachment was monitored with a microscope and trypsin digestion was stopped by adding 9 mL of MEM containing 10% FBS. An inoculum was transferred to a new 75 mL tissue culture flask. Inoculum size varied between 1:15 to 5:15 inoculum:MEM, considering the viable cell numbers and the experiment schedule.

3.2.9 Invasion assay

The anti-invasive activity of each fraction/component was assessed by a gentamicin protection invasion assay on HeLa cells between passages 1-20 using exponential phase *S.* Typhimurium SL1344 as challenge bacteria (Sharon Ruschkowski, Biotechnology Laboratory UBC, personal communication, 1997).

A loopful of bacteria was cultured in 2 mL of antibiotic-free LB broth in a sterile borosilicate screw cap tube (20x 150 mm VWR scientific, Mississauga, ON) and grown without shaking for 16 hours, at 37°C. Confluent HeLa cells were trypsinized and their population density was adjusted to 10^5 cells per mL of MEM. The sterile 24 well tissue culture plates were seeded with 1 mL of suspended HeLa cells and incubated for 18 hours at 37°C in humidified 5%: 95%, CO_2: air incubator

to obtain sub-confluency the next day. One day old *S.* Typhimurium culture was subcultured at 3:100 dilution in pre-warmed LB broth and grown for 3 hours at 37°C to obtain the bacteria in exponential phase. Thirty minutes before the bacterial culture was ready, old MEM was removed from the cultured HeLa cells in the tissue culture plate wells by vacuum suction. MEM containing the test samples (300 μL) was then added to each well. MEM with no added protein was used as negative control. The test compounds were filter sterilized by low protein binding 0.2 μm Acrodisc® syringe filters (catalogue number 4454, PALL Gelman Ltd, Ann Arbor, MI USA) and allowed to incubate with cultured cells for 30 minutes followed by the addition of 5 μL of the challenge bacterial suspension (~ 5 x 10^5) into the HeLa cell culture-test protein mixture.

The population of *S.* Typhimurium was determined for each inoculum by the drop plate counting method (ICMSF, 1978; Facon and Skura, 1996). *S.* Typhimurium were allowed to invade HeLa cells for 30 minutes (Sharon Ruschkowski Biotechnology laboratory, UBC, personal communication, 1997) then cells were washed two times with PBS^{++} to remove unbound or loosely bound extracellular bacteria. HeLa cells were then incubated for 90 min with 500 μL of 100 μg gentamicin (Sigma, G1272) per mL MEM to kill the extra-cellular *S.* Typhimurium. HeLa cells were washed with PBS^{++} to remove any antibiotic residues. Internalized *S.* Typhimurium were released by disrupting the HeLa cell membrane with 1 mL of filter sterilized PBS containing 1% (v/v) Triton X-100 and 0.1% (w/v) sodium dodecyl sulphate (SDS). The cell lysates were 1:10 serially diluted in PBS^{--} and

cultured in duplicate on LB agar plates by the drop plate method (Facon and Skura, 1996). Plates were incubated for 24 hours and bacterial colony forming units (CFU) were enumerated and compared to negative control. Results were recorded as percentage invasion compared to the negative control. Percentage invasion inhibition by whey proteins was calculated using the following formula:

Percentage invasion inhibition = $100-[(CFU_{\text{protein treated}}/CFU_{\text{control}}) \times 100]$

where control contained extra MEM instead of test samples.

Experiments were conducted in a level II biosafety laboratory and a class II cabinet (Bio-Klone 2, BK-2-6 Microzone Inc. Nepean, ON). FBS, which by preliminary experiments did not show any anti-invasive activity against *S.* Typhimurium, was used as another control. Figure 3.1 illustrates the simplified flow diagram of the invasion assay.

3.2.10 Cytotoxicity of whey proteins to HeLa cells

Confluent HeLa cells were trypsinized and their population density was adjusted to 10^5 cells per mL. A 24 well tissue culture plate was seeded with 1 mL of cell suspension per well and incubated at 37°C in 5% CO_2, 95% air to allow attachment and sub-confluency. After 18 hours of incubation, old medium was removed from the tissue culture plate and fresh MEM containing the test proteins was added to each well. MEM-test samples were filter sterilized prior to use. After incubation for 60 minutes (the same time required in treating the HeLa cells in the actual invasion

assay) HeLa cells were washed with 1 mL of PBS^{++} and trypsinized using 30 μL of trypsin-EDTA. Viable cells were detected by the trypan blue dye exclusion method and counted by microscopy (WILD Heerbrugg, Switzerland). Aliquots of 50 μL of suspended cells were placed in a 1 mL centrifuge tube and 50 μL of 0.4% trypan blue solution (Sigma T8154) was added and mixed. After 1-2 minutes, a 20 μL sample was taken and introduced to a Neubauer hemocytometer covered with standard hemocytometer coverslip. To prevent sampling error, cells were resuspended every time before taking the samples since the cells have a tendency to re-attach to the container surface. Numbers of viable cells were counted in 8 grid squares of the hemocytometer and compared to non-protein treated HeLa cells, which were used as negative control.

3.2.11 Statistical Analysis

All experiments were independently repeated 3 times with 3 replicates each time unless otherwise stated. The results were recorded as mean values. Statistical analysis to determine the significance of difference ($p< 0.05$) between mean values of each test and control was performed using paired t-test in MINITAB 13.31 for Windows (MINITAB™, statistical software Minitab Inc. State College PA USA).

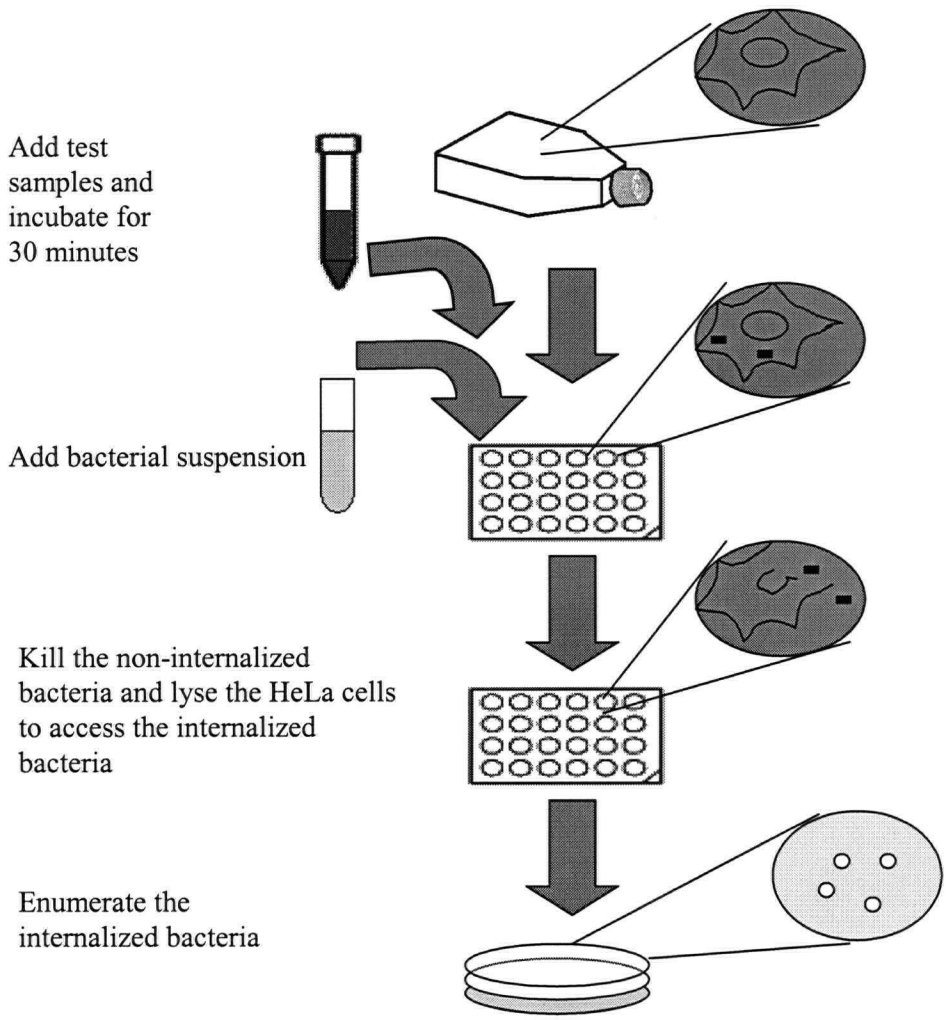

Figure 3.1. Simplified flow diagram of invasion assay

3.3 Overview of work plan

3.3.1 Evaluation of anti-invasive activity of bovine colostrum and non-IgG fraction

Bovine colostrum has been reported to have anti-bacterial activity against a wide variety of microorganisms (Uruakpa, Ismond and Akobundu, 2002). The role of immunoglobulins in specifically inhibiting the pathogens has been studied and extensively reported. Most research studies on anti-bacterial activity of food components have mainly focused on the CFU comparisons of treated and non-treated samples. Although the information obtained can be useful, there is always a chance of biased conclusions due to unexpected situations such as clumping of the bacterial cells, which could produce a false lower CFU value as a consequence of exposure to the test material. To date there has not been much research on the effect of anti-bacterial agents in food systems using more complex techniques such as cell culture.

The present study was designed to investigate the effect of bovine colostrum and its IgG–free fraction on invasion of HeLa cells by *S.* Typhimurium. Colostrum samples were collected throughout the year and pooled. Colostral whey was prepared by acid precipitation of casein of the skimmed collected bovine colostrum. IgG was separated from the skimmed colostral whey using protein G affinity column chromatography. SDS-PAGE and ELISA were conducted to ensure the separation of IgG. Commercial bovine IgG was used as control. Aliquots of 50 μL of colostral whey, IgG-free fraction of bovine colostral whey and commercial bovine IgG, prepared in 200 μL of MEM, were subjected to HeLa cell invasion assay using

exponential phase *S.* Typhimurium as challenge bacteria. The results were compared to negative control. The simplified flow diagram of described procedures is presented in Figure 3.2.

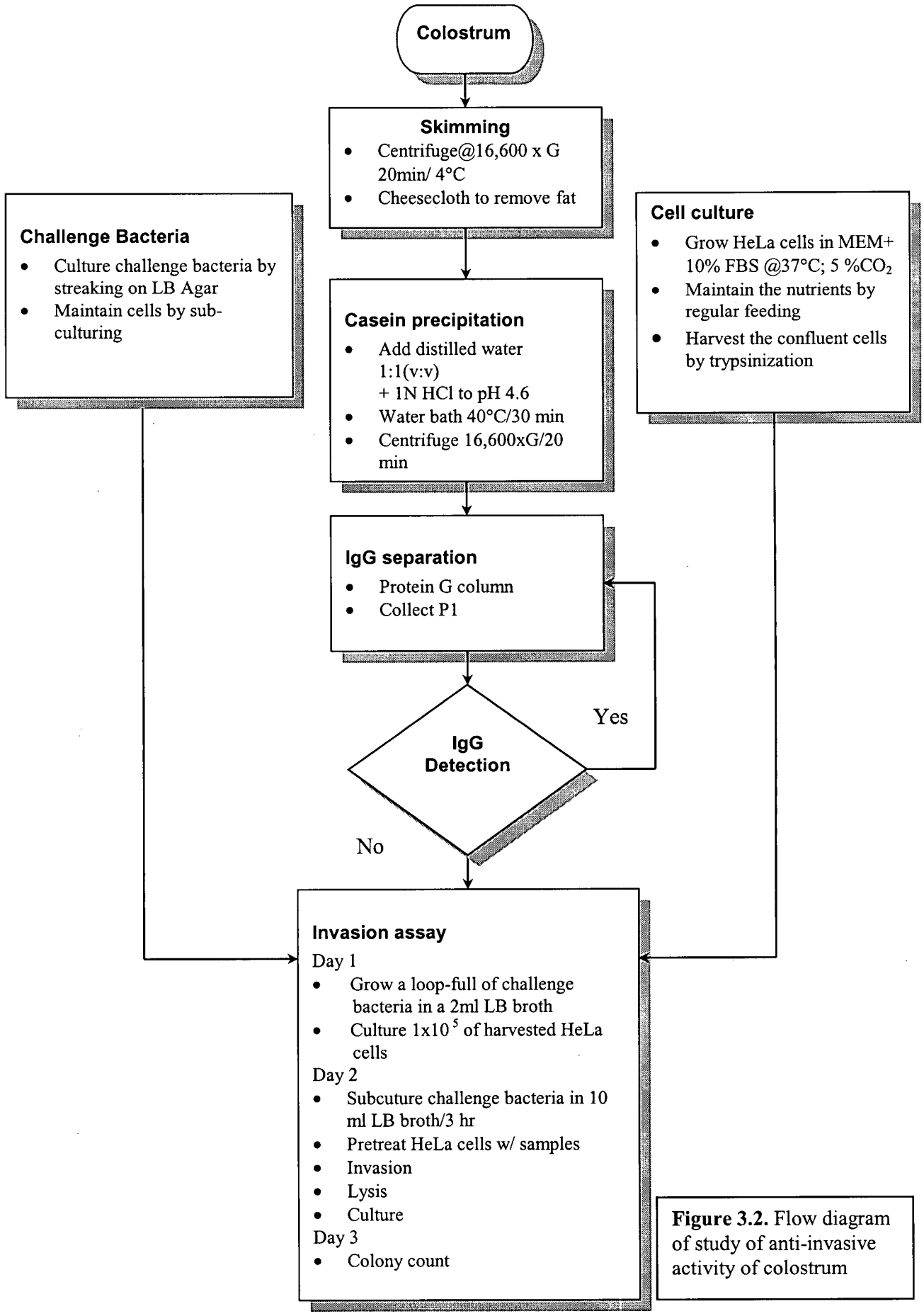

Figure 3.2. Flow diagram of study of anti-invasive activity of colostrum

3.3.2 Evaluation of anti-invasive activity of whey proteins

To determine the colostral whey component(s) contributing to the anti-invasive activity of the IgG-free fraction, several whey proteins were selected and tested for their anti-invasive activity. The selection was made based on either their abundance in bovine whey or previously reported anti-bacterial effect. α-Lactalbumin, β-lactoglobulin, lactoferrin and lactoperoxidase were selected and tested for their anti-invasive activity at the following concentrations:

Lactoferrin (6.3 mg mL^{-1}); α-lactalbumin (8 mg mL^{-1}); β-lactoglobulin (5.2 mg mL^{-1}); β-lactoglobulin A (5.2 mg mL^{-1}); β-lactoglobulin B (5.2 mg mL^{-1}) and lactoperoxidase (1.6 mg mL^{-1})

These concentrations were chosen based on the manufacturer's data and were the concentration used for quality assurance evaluation of the whey proteins (Sigma technical service, personal communication, 1999).

To have conclusive results, it was important to ensure that the tested whey proteins were not toxic to HeLa cells at the concentrations used. A cytotoxicity test was conducted by checking the viability of HeLa cells after exposure to whey proteins using the trypan blue dye exclusion method. Cell morphology was also monitored with a microscope. Figure 3.3 shows the simplified flow diagram of the described procedures.

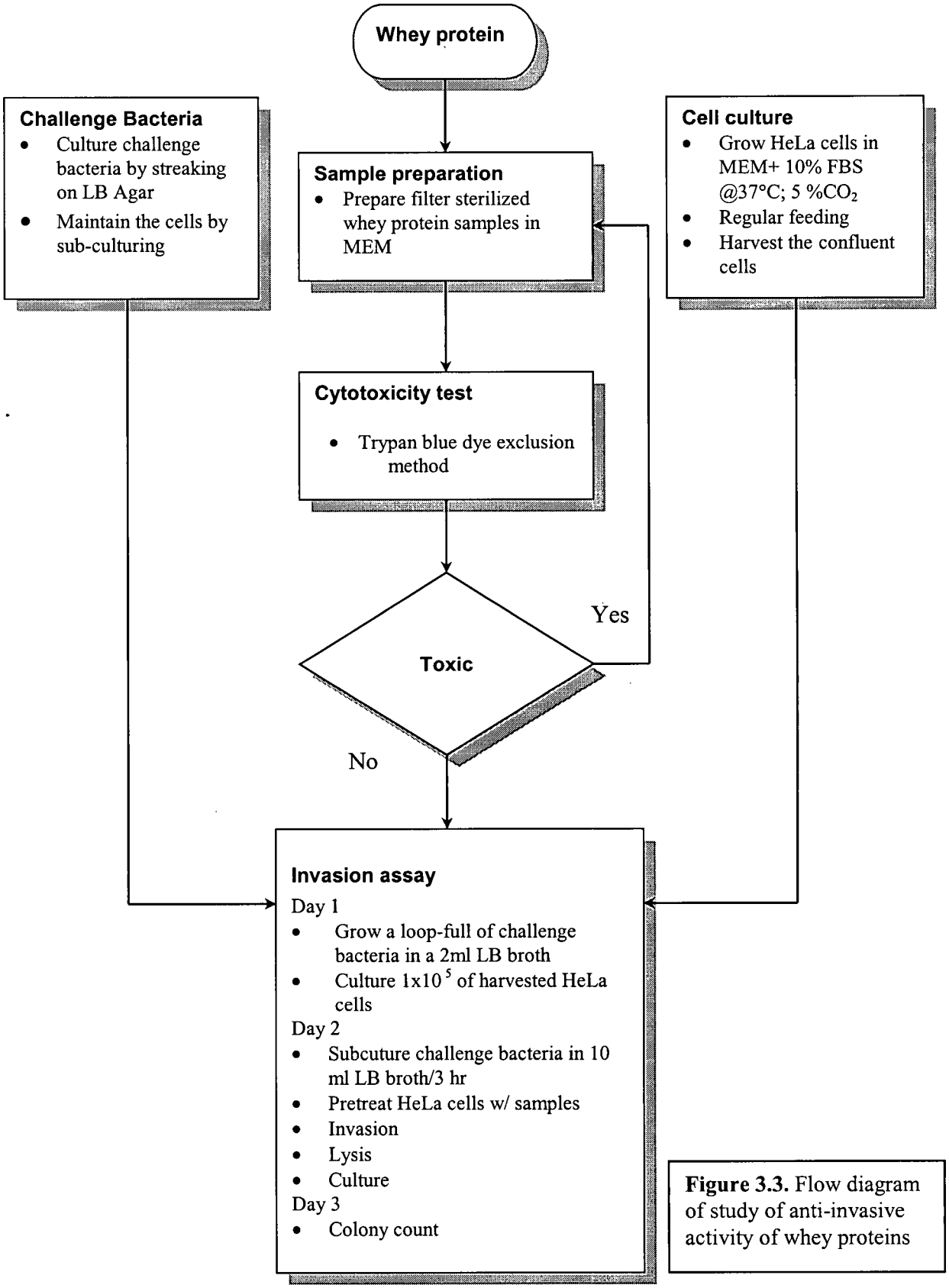

Figure 3.3. Flow diagram of study of anti-invasive activity of whey proteins

3.4 Results and discussion

The ability to penetrate the intestinal epithelium is an important virulence factor in pathogenesis of *S.* Typhimurium. The objective of the experiments reported in this chapter was to confirm the anti-invasive activity of bovine colostrum against *S.* Typhimurium using HeLa cell culture technique and also to investigate the effect of the IgG-free fraction of bovine colostral whey and selected whey proteins on invasion of HeLa cells by *S.* Typhimurium.

3.4.1 Separation of immunoglobulin G from bovine colostrum

The specific anti-bacterial activity of immunoglobulins has been studied extensively and discussed elsewhere in this thesis. To determine the effect of other factors contributing to the overall anti-bacterial property of colostrum, the first step was to separate the IgG from colostrum. Several methods have been described for separation of IgG from its sources. To name a few, ultrafiltration combined with immobilized metal affinity chromatography or gel filtration has been reported to separate immunoglobulins with different purity levels (Al-Mashikhi, Li-Chan, and Nakai 1988; Kanamaru, Ozeki, Nagaoka, Kuzuya and Niki, 1993; Fukumoto, Li-Chan, Kwan and Nakai, 1994). All procedures require a final stage of affinity chromatography to achieve a high purity immunoglobulin. Protein G, obtained from group G streptococci has demonstrated a high affinity for binding to the Fc region of different subclasses of immunoglobulin G from a variety of mammals (Guss *et al.*,

1986). Based on this fact, protein G-agarose affinity columns have been developed to isolate IgG.

Skimmed colostral whey samples were applied to a protein G-agarose column, bound and unbound fractions were separated using buffers with different pH values (see section 3.2.1.1). Flow rate has an important impact on binding of IgG to the column. Preliminary experiments conducted to find the best flow rate for washing and eluting, showed that binding buffer flow rates higher than 0.27 mL min^{-1} resulted in IgG appearing in the unbound fraction. A typical elution profile from the protein G column elution is shown in Figure 3.4. The elution diagram consisted of two peaks, which correspond to unbound (P1) and bound (P2) fractions. The respective fractions of each peak were pooled and used in subsequent assays. The pooled unbound fraction was subjected to SDS-PAGE and ELISA to ensure complete separation of IgG. Figure 3.5 illustrates the results of SDS-PAGE under reducing conditions followed by coomassie blue staining. Lane 1 shows the protein pattern of skimmed, decaseinated bovine colostrum. Commercial IgG in lane 2 showed bands around 25 kD and 55 kD regions. The bands correspond to light chain and heavy chain of IgG respectively. Lane 3 of the SDS-PAGE gel shows that the pooled eluted fractions of peak 1 of the protein G affinity column did not contain 25 kD and 55 kD bands corresponding to the presence of IgG. Although confirmation requires more specific testing, it can be speculated that IgG was removed from colostral whey by protein G affinity chromatography. Bands appearing at 14, 18, 66-84 kD region of the unbound fraction (lane 4) can be attributed to the presence of lysozyme and/or α-lactalbumin,

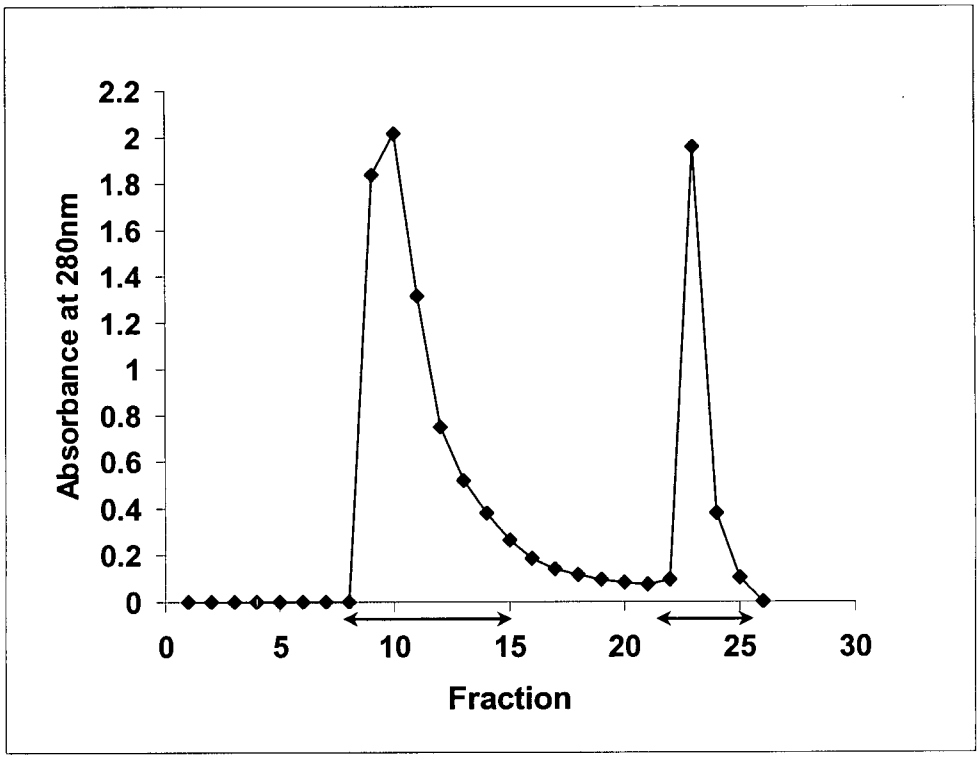

Figure 3.4. Elution pattern of non-IgG (P1) and IgG (P2) fractions of bovine whey colostrum from a protein G- agarose affinity column equilibrated and washed with sodium acetate (binding) buffer. The bound fraction (P2) was eluted by glycine-HCl buffer. Elution was monitored at 280 nm. Arrows underneath each peak correspond to fractions which were pooled.

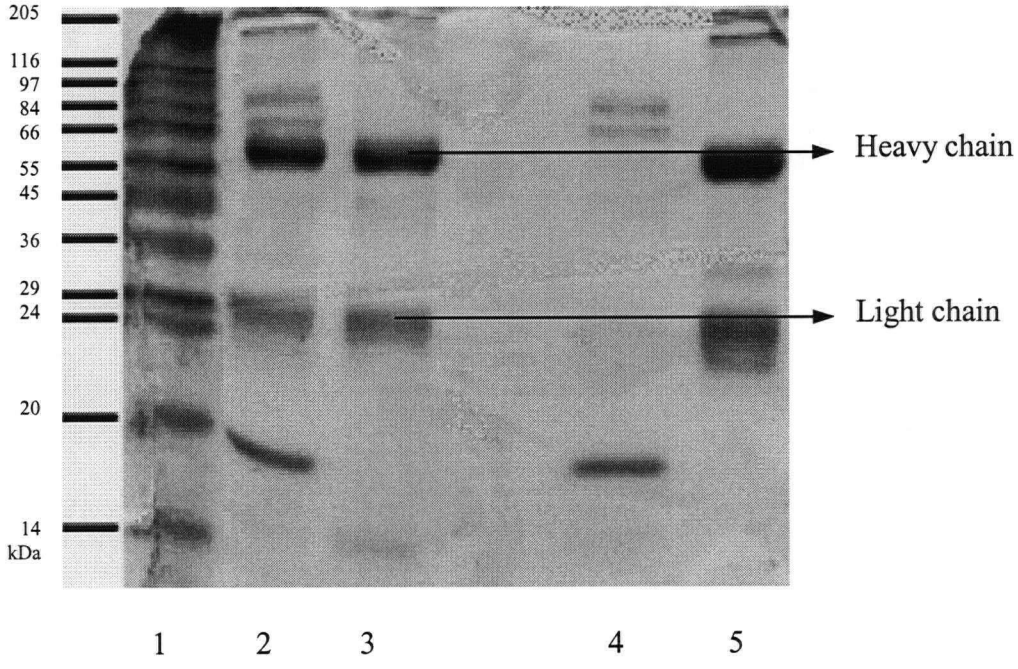

Figure 3.5. Reducing SDS-PAGE profiles of skimmed colostral whey before and after protein G chromatography.
Protein Marker (1); Skimmed bovine colostrum whey (2); commercial IgG (3); pooled fractions of P1, the peak eluted with acetate buffer (4) and P2, the peak eluted with glycine-HCl buffer (5) collected after passing through protein G-agarose column.

β-lactoglobulin, lactoferrin and/or bovine serum albumin and lactoperoxidase respectively. Lane 5, the bound fractions (P2) from the affinity column, showed bands in 50 and 25 kD region, which can be attributed to IgG heavy chain and light chain respectively.

The results of SDS-PAGE show that de-caseinated colostral whey was successfully fractionated into two fractions of IgG and non-IgG through protein G affinity column chromatography.

Sandwich ELISA, a sensitive method for determination of a broad range of biochemical substances, is a powerful analysis method (Sauer and Foulkes, 1983; Kummer *et al.*, 1992). Sandwich ELISA was conducted on pooled fractions of peak 1 to detect the presence of IgG and evaluate the efficacy of protein G column in separating IgG. ELISA did not show any detectable levels of IgG in pooled fractions of peak 1. Since the sensitivity of ELISA was reported to be 1 ng IgG per mL milk (Losso, Kummer, Li-Chan and Nakai, 1993) ELISA results confirmed the separation of IgG from colostral whey after protein-G agarose affinity chromatography.

3.4.2 Anti-invasive activity of bovine colostrum and its IgG-free fraction

A bacterial growth curve was established to determine the required incubation time to reach exponential phase. Under the conditions in the study, *S.* Typhimurium reached exponential phase after 3 hours of static incubation at 37°C (data not shown). This incubation time was used to produce *S.* Typhimurium culture for the following invasion assays.

Whole colostrum, pooled fraction of peak 1 and commercial IgG were subjected to the gentamicin protection invasion assay with HeLa cell monolayers using exponential *S.* Typhimurium as challenge bacteria. Peak 2 (IgG) was not tested since Facon (1995) clearly demonstrated anti-invasive activity in the IgG fraction (peak 2) from numerous samples of colostrums and milk whey. Facon (1995) showed anti-invasive activity in the unbound fraction from protein G column but did not characterize the active component(s). The assay is based on the fact that gentamicin is unable to penetrate eukaryotic cells (Antonini *et al.*, 1997). Applying gentamicin to HeLa cells at the end of the assay kills the extracellular bacteria without affecting the intracellular bacteria. Lysing the HeLa cell monolayers gives access to internalized bacteria, which can be quantified by direct plating. HeLa cells treated with bovine whey colostrum showed 98% reduction in *S.* Typhimurium invasion (Figure 3.6). The results are in agreement with the work done by Facon (1995) in which bovine colostrum reduced the invasion of S. Typhimurium by 87-97%. Numerous studies have shown protective effects of human milk and bovine milk immunoglobulins against pathogens (Glass *et al.*, 1983; Cruz, Gil, Cano, Caceres and Pareja, 1988; Cravioto, Tello, Villafan, Ruiz, Vedovo and Neeser, 1991; Camara, Carbonare, Silva and Carneiro-Sampaio, 1994). Potential anti-bacterial activity of casein and its fractions has recently been the subject of several studies. Human milk casein did not show any anti-bacterial effect at 300 $\mu g\ mL^{-1}$ concentration (Araujo and Giugliano, 2001). Although the anti-bacterial activity of milk and colostrum is usually attributed to the naturally present immunoglobulins (Korhonen, 2000), we found that the pooled

fraction of peak 1 (P1) also was effective in inhibiting *S.* Typhimurium invasion of HeLa cells by 65%. Since electrophoretic analysis and immunoassay did not detect any IgGs in P1 and considering the fact that the sensitivity of ELISA is 1 ng mL^{-1} it can be concluded that IgG was not responsible for the observed anti-invasive activity in the unbound fraction of colostral whey after passing through a protein G column. Ultimately, the finding suggests that there are factors, other than IgG, which also contribute to the total anti-invasive activity of colostrum. Similar results were reported with human milk. Araujo and Giugliano (2000) showed that non-immunoglobulin fraction of human milk inhibited adherence of Diffusely Adherent *Escherichia coli* (DAEC) and enteroaggressive *Escherichia coli* (EAEC) to HeLa cells. Ashkenazi and Mirelman (1987) found that the non-immunoglobulin fraction of human milk decreased adhesion of *E. coli* to guinea pig intestinal tract. They also found 50% anti-adherence activity in strains possessing colonization factor antigen (CFA) while no inhibition was noted in strains lacking CFA.

In the present study, commercial serum IgG did not decrease the *S.* Typhimurium invasion of HeLa cells. Since IgG specifically inhibits the invasion phase, one speculation would be that the cows from which the commercial IgG was obtained were not in contact with *S.* Typhimurium.

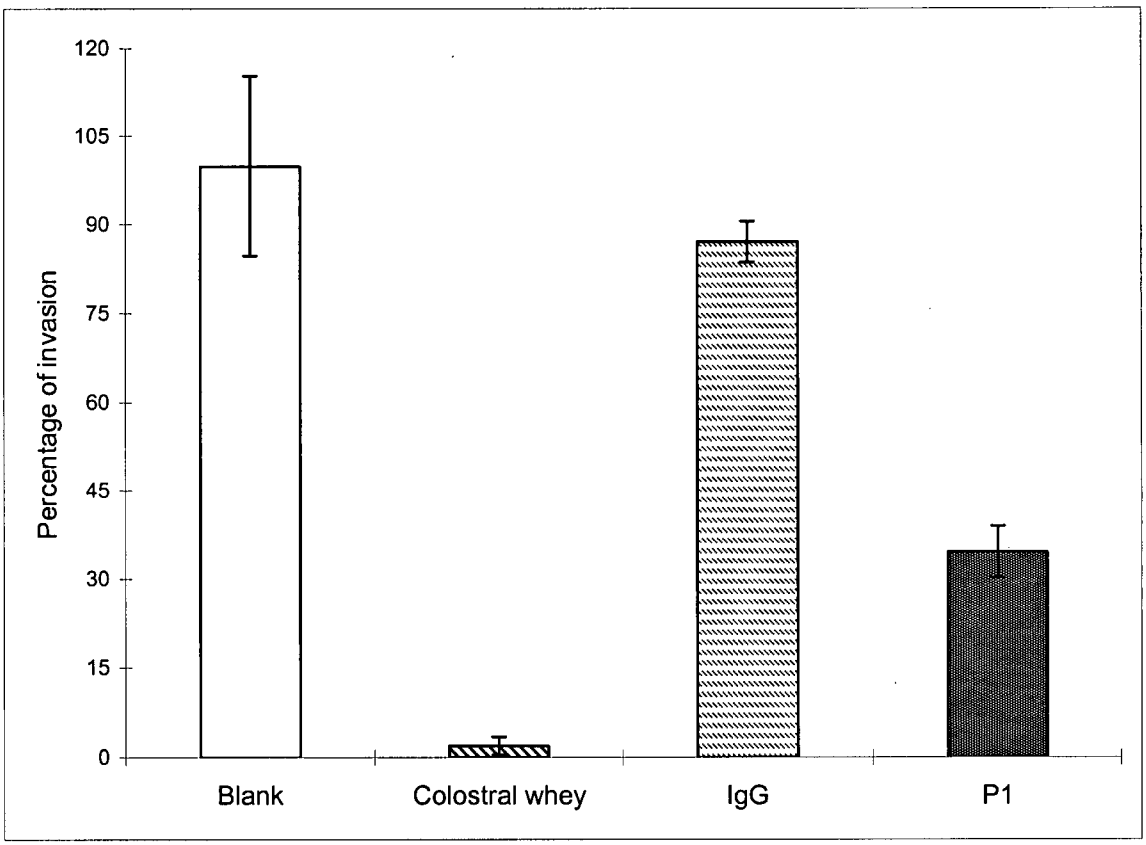

Figure 3.6. Percentage of internalized *S.* Typhimurium after incubation of HeLa cells with test samples, relative to control. Values represent the average of triplicates from three independent trials. Bars on each column represent standard deviations.
Blank: HeLa cells with no added protein; Colostral whey: Skimmed, casein-free colostrum; IgG: Commercial immunoglobulin G (IgG); P1: Pooled immunoglobulin G-free fraction after passing colostrum through protein G-agarose column.

3.4.3 Cytotoxicity of whey proteins

Cytotoxicity test was conducted to determine if the whey proteins at the concentrations used had any adverse effect on HeLa cell integrity. The method is based on the ability of viable cells to exclude trypan blue stain from permeating through the cell membrane (Martin, 1994). Whey proteins were dissolved in MEM at 10 mg mL^{-1} and incubated with HeLa cells for 60 minutes. Viability of cells after exposure to whey proteins was tested by detaching the protein-treated cells using trypsinization following trypan blue dye treatment and counting the viable cells. Preliminary experiments showed that a prolonged procedure might result in inaccurate enumeration of HeLa cells due to their tendency for attachment to the tube surface so it was important to test each sample individually or a maximum of two samples at a time. The viabilities of HeLa cells exposed to whey proteins were compared to non-treated HeLa cells grown under the same conditions. No significant difference ($p>0.05$) was found between the number of viable cells in whey protein-treated and non-treated HeLa cell cultures indicating that the proteins tested were not cytotoxic to HeLa cells at the concentration and duration used (Table 3.1). Matin, Mizumoto and Otani (2001) tested the cytotoxicity of α-lactalbumin by incubating bovine, human and goat α-lactalbumin with mouse spleen cells. They found that all α-lactalbumin samples were cytotoxic to mouse spleen cells at 500 μg mL^{-1}. They attributed this cytotoxicity to conformational changes of α-lactalbumin and its calcium binding activity. Carboxymethylated α-lactalbumin, which does not have the ability to bind calcium, was not cytotoxic (Matin *et al.*, 2001). Cytotoxicity of

Table 3.1. Evaluation of cytotoxicity of whey proteins (10 mg mL^{-1}) on HeLa cells

Whey proteins	*Treated* $N \times 10^4$ *cells*	*Non-treated* $N \times 10^4$ *cells*
La	21.6 (4.4)	21.2 (2.39)
Lg	19.4 (2.2)	19.8 (3.11)
Lf	23.2 (2.59)	24.4 (2.79)

La: α-lactalbumin, Lg: β-lactoglobulin, Lf: lactoferrin
Values represent average of 3 replicates. Paired t-test was conducted on values from treated and non-treated samples
Numbers in brackets are standard deviations

multimeric human α-lactalbumin to tumor cells has been reported while monomeric α-lactalbumin was not cytotoxic (Håkansson, Zhivotovsky, Orrenius and Sabharwal, 1995). Also it has been reported that different cell lines have different sensitivity to multimeric α-lactalbumin (MLA). While mouse leukemia cells lost 50% viability after 6 hours of exposure to 500 μg mL^{-1} MLA, primary kidney epithelial cells retained full viability after 24 hours of exposure to 4 mg mL^{-1} of MLA (Håkansson, Anderasson, Zhivotovsky, Karpman, Orrenius and Svanborg, 1999).

The non-cytotoxicity of the α-lactalbumin used in our experiment could be the result of monomeric conformation or the resistance of HeLa cells to α-lactalbumin at the concentration used and duration of exposure.

Any cell loss due to cytotoxicity during the experiment would result in lower numbers of internalized bacteria, which in turn would lead to false conclusion. In the present study, the cytotoxicity and morphology tests were conducted in order to rule out the possibility of cell loss due to exposure to whey proteins during the invasion assay. Microscopy of cultured cells incubated with whey proteins did not show any morphology changes.

3.4.4 Anti-invasive activity of whey proteins

Based on the results of the anti-invasive activity of peak 1 (IgG-free), it was decided to determine the component(s) in IgG-free fraction of colostrum whey, which contributed to the observed anti-invasive activity. Two approaches were considered for finding the active factor(s). The first approach was to use sequential

chromatography and conducting the invasion assay on each fraction and the other one was to use the already known and purified whey components and test their anti-invasive activity against *S.* Typhimurium. Considering the fact that using pure components would be more time, cost and energy efficient and also to avoid the complexity of using sequential chromatography, it was decided to initially work on the already known and purified whey proteins and investigate their ability to inhibit the *S.* Typhimurium invasion of HeLa cells. The second approach was reserved in case the first approach was not successful in finding active component(s).

The criteria for selection of whey proteins were either abundance or reported anti-bacterial activity. α-Lactalbumin and β-lactoglobulin were selected for being major whey proteins and lactoperoxidase and lactoferrin were chosen because of their reported anti-bacterial activity. Lactoperoxidase was reported to show anti-microbial activity when it is used along with thiocyanate and hydrogen peroxide. We were curious to find out if lactoperoxidase apart from the lactoperoxidase system has an effect on inhibition of *S.* Typhimurium invasion of HeLa cells. Figure 3.7 shows the reducing SDS-PAGE profile of the mentioned whey proteins. The purity of the proteins is as follows: α-Lactalbumin, 85%; β-lactoglobulin, 90%; β-lactoglobulin A, 90%; β-lactoglobulin B, 90%; lactoferrin, 90% and lactoperoxidase 90% (Manufacturer's data, Sigma).

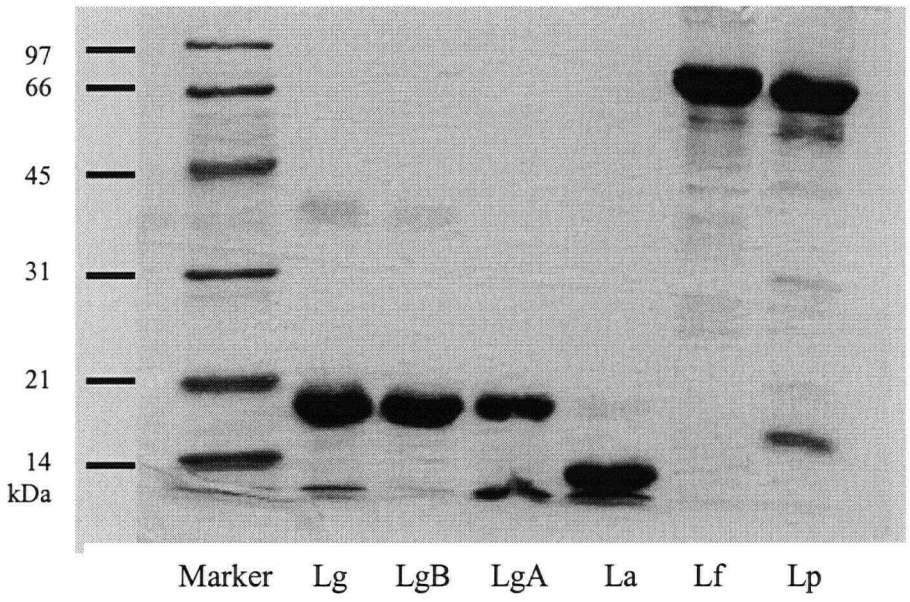

Figure 3.7. Reducing SDS-PAGE on selected whey proteins
Lg: β-lactoglobulin, LgB: β-lactoglobulin B, LgA: β-lactoglobulin A, La: α-lactalbumin, Lf: lactoferrin, Lp: lactoperoxidase Low range molecular weight markers were used as standard (BioRad 161-0304). Total protein content for each whey protein was adjusted to 5 μg.

A gentamicin protection assay was used to evaluate the potential anti-invasive activity of these proteins. Results show that lactoferrin, α-lactalbumin, β-lactoglobulin (mix; variants A and B) decreased the invasion of HeLa cells by *S.* Typhimurium, while lactoperoxidase had minimal effect (Figure 3.8). The highest inhibition was observed with β-lactoglobulin A.

Among the whey proteins, the anti-bacterial activity of lactoferrin and lactoperoxidase have been extensively studied and will be discussed in chapters 4 and 5 in more detail. Use of cell culture techniques has recently opened the way for studying anti-bacterial agents. To the writer's knowledge there are only a few reports on anti-bacterial activity of whey proteins as it pertains to anti-invasive or anti-adhesive properties. Lactoferrin and its hydrolysates have been the focus of most of the reported research. Lactoferrin at 1 mg mL^{-1} decreased the number of internalized *Listeria monocytogenes* in HT-39 and Caco-2 cells by 42-125 fold (Antonini *et al.*, 1997). In another study, bovine lactoferrin at 2 mg mL^{-1} did not prevent invasion of HeLa cells by *Escherichia coli* (Longhi, Conte, Seganti, Polidoro, Alfsen and Valenti, 1993). β-Lactoglobulin inhibited the adhesion of *Escherichia coli* HB101 and *Klebsiella oxytoca* to human ileostomy glycoproteins (Ouwehand, Salminen, Skurnik, and Coway, 1997).

Another interesting point in our results was the difference found in anti-invasive activity of variants A and B of β-lactoglobulin. These two variants have a structural difference of 2 amino acid residues. Variant B has glycine in position 64 instead of aspartic acid and alanine in postion 118 versus valine in variant A (Le Bon,

Durand and Nicolai, 2002). Structural examination of these two variants shows the presence of three carboxyl groups in the vicinity of the aspartic acid residue at position 64 of variant A. It has been proven that octamerization of β-lactoglobulin molecule occurs at this site. Protonation studies show binding of one proton per monomer in variant B versus two protons per monomer in variant A (Whitney, 1988). Substitution of tryptophan with tyrosine (less hydrophobic) in a peptide derived from lysozyme eliminated bactericidal activity (Pellegrini, Thomas, Bramaz, Klauser, Hunziker and von Fellenberg, 1997). There are some theories that hydrophobic properties and expressing positive charge at neutral pH are important characteristics for bactericidal peptides. Gram-negative bacteria have negatively charged lipopolysaccharide in their outer membrane. Thus positively charged peptides are more effective against Gram-negative bacteria (Pellegrini, Thomas, Bramaz, Klauser, Hunziker and von Fellenberg, 1999). In another study, Pellegrini and coworkers (2001) showed that negatively charged peptides from β-lactoglobulin were bactericidal against Gram-positive bacteria. Considering this theory, in the present study there should have been less anti-invasive activity with β-lactoglobulin A since glycine is a neutral amino acid while aspartic acid is negatively charged. β-Lactoglobulin A, however, was more efficient as an anti-invasive agent (Figure 3.8).

In a study on α-lactalbumin peptides, researchers found that changing a single amino acid in the polypeptide chain (replacing leucine with isoleucine which is more hydrophobic) diminished the bactericidal potency of the polypeptide (Pellegrini *et al.*, 1999). Their findings suggest that hydrophobicity did not play a role in conferring the

bactericidal effect in the peptides they tested. As cited by Nakai and Li-Chan (1988) there are disagreements among scientists about the magnitude of hydrophobicity of leucine and isoleucine and the reported values depend on the method used to evaluate the hydrophobicity of amino acids.

It seems that although hydrophobicity and electrical charge are important characteristics for bactericidal activity of agents, which interfere with bacterial membrane (Pellegrini *et al.*, 1999), the overall structure of the bactericidal peptide is also important and needs to be considered. The importance of hydrophobicity and electrical charge on anti-invasive activity of proteins and peptides needs to be investigated.

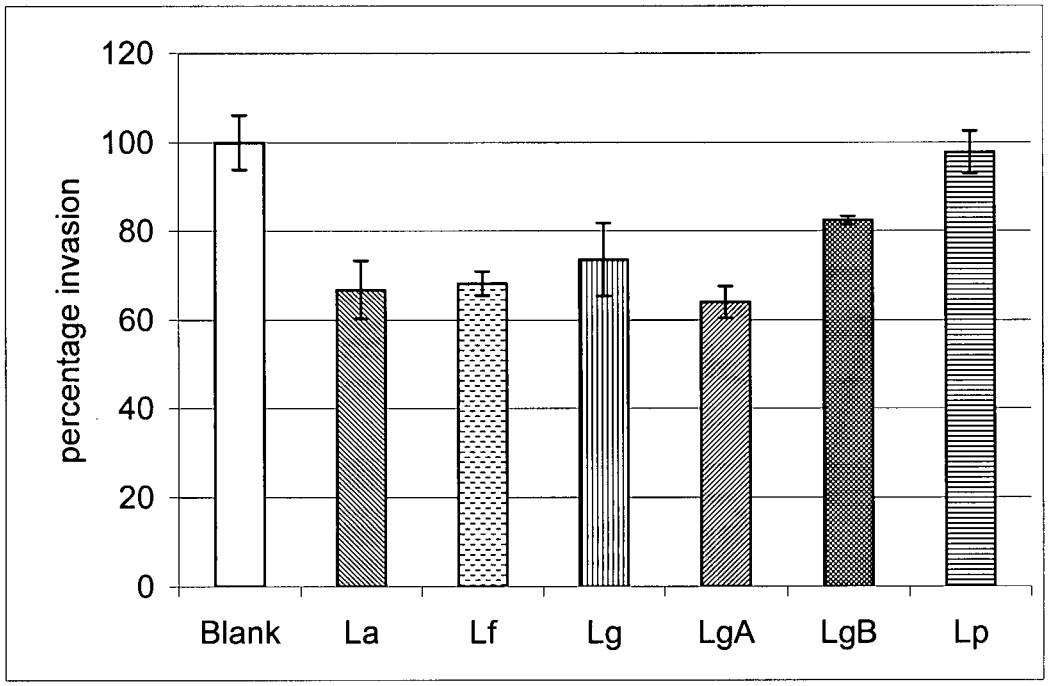

Figure 3.8. Percentage of internalized *S.* Typhimurium after incubation of HeLa cells with whey proteins, relative to control. Values represent the average of triplicates from five independent trials. Bars on each column represent the standard deviation Blank: HeLa cells with no added protein; La: α-lactalbumin (8.0 mg mL^{-1}); Lf: Lactoferrin (6.3 mg mL^{-1}); Lg: β-lactoglobulin (5.2 mg mL^{-1}); Lg A: β-lactoglobulin variant A (5.2 mg mL^{-1}); Lg B: β-lactoglobulin variant B (5.2 mg mL^{-1}); Lp: Lactoperoxidase (1.6 mg mL^{-1})

In the present study, the concentrations of whey proteins were chosen based on the manufacturer's data and were the concentration used for quality assurance evaluation of the whey proteins (Sigma technical service, personal communication 1999). The whey protein concentrations were not equal. This inequality makes it difficult to compare the effect of concentration on anti-invasive activity of whey proteins. To overcome this problem, the percentage of anti-invasive activity per mg protein used was calculated. β-Lactoglobulin A, β-lactoglobulin mix and lactoferrin had the highest anti-invasive activity per mg protein amongst the proteins tested (Table 3.2).

Table 3.2: Anti-invasive activity of whey proteins per mg of protein

	%Anti-invasive activity	*% Anti-invasive per mg protein**
La	33 (6.52)	4.11
Lf	32 (2.71)	5.04
Lg	26 (8.06)	5.00
LgA	36 (3.60)	6.92
LgB	18 (0.95)	3.46
Lp	2 (4.77)	1.27

La: α-lactalbumin (8 mg mL^{-1}), Lf: lactoferrin (6.3 mg mL^{-1}); Lg: β-lactoglobulin (5.2 mg mL^{-1}), LgA: β-lactoglobulin A (5.2 mg mL^{-1}), LgB: β-lactoglobulin B (5.2 mg mL^{-1}), Lp: Lactoperoxidase (1.6 mg mL^{-1})
Values represent average of 3 replicates from 5 independent trials (n=15)
Numbers in brackets represent standard deviations.
* % Anti-invasive activity per mg protein was calculated by dividing the percentage of anti-invasive activity by the mg protein used.

3.5 Conclusion

Work reported in this chapter opened an avenue for further investigation of anti-invasive activity of milk and colostrum components. In this research we described using HeLa cell culture to study the anti-invasive activity of whey proteins against *S.* Typhimurium. As measured by gentamicin protection assay, we have presented evidence that bovine colostrum can successfully inhibit the invasion of HeLa cells by *S.* Typhimurium. Also we proved that non-immunoglobulin fraction of bovine colostrum and individual whey proteins contribute to this anti-invasive activity. Questions regarding the concentration dependence of anti-invasive activity and mechanism of inhibition remain to be answered.

Our results suggest that bovine whey proteins not only serve as a nutrition supply for human but also may provide protection against invasion by *S.* Typhimurium. This characteristic can be taken into account in formulating novel foods with functional properties.

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CHAPTER FOUR

Effect of selected whey proteins, individually and as mixtures on S. Typhimurium invasion of HeLa cells

4.1 Introduction

In formulating any product it is important to determine the required amount of each component to fulfil the objective for which the formulation is made. This is vital when the bioactivity of certain component(s) is the main purpose of formulating such a mixture. In pharmaceutical/nutraceutical sciences, formulation of products require dose-response investigations, which involve finding the optimum level of bioactive compounds within a concentration range that is safe for application.

Results of our earlier studies confirmed the anti-invasive activity of bovine colostrum and we demonstrated that non-immunoglobulin G (non-IgG) fraction of colostrum whey contributes to the anti-invasive property of bovine colostrum. To elucidate the active component(s) responsible for conferring anti-invasive activity in non-IgG fraction, the approach selected was to evaluate known proteins in colostrum whey for their anti-invasive properties. Some non-IgG whey proteins and their peptides have been reported to have antimicrobial activity amongst which, lactoferrin and lactoperoxidase are the most studied compounds. The antimicrobial activity of whey proteins was thoroughly reviewed by Naidu (2000) and based on such information we decided to use whey proteins in our investigation.

Most of the reviewed sources used the bacterial growth inhibition method to investigate the anti-bacterial properties of lactoferrin and lactoperoxidase. As stated elsewhere in this thesis, this method has shortcomings for evaluating anti-bacterial activity. At the time of writing this thesis, while there were limited reports on anti-invasive activity of lactoferrin, whey proteins were not specifically investigated for

their anti-invasive property against *S.* Typhimurium using HeLa cell culture invasion assay.

Considering the complex nature of food, interaction between its constituents is inevitable. Understanding these interactions is important, if not critical, in formulating a new food product especially when the functionality or biological activity of certain constituent(s) is concerned. The interactions may be additive, synergistic or antagonistic. In any case, it is important to determine the existence of such interactions amongst the components.

The present study was initiated with the objective of investigating the effect of whey protein concentration (individually and in the mixture) on inhibition of *S.* Typhimurium invasion of HeLa cells and also to formulate a mixture of whey proteins with high potency anti-invasive activity.

4.2 Materials and Methods

4.2.1 Whey proteins

Bovine lactoferrin (L4765), α-lactalbumin Type III (L6010) β–lactoglobulin 3X crystallized (L0130), and lactoperoxidase (L2005) were purchased from Sigma Chemicals (St Louis, MO. USA). (For details of the procedures for determination of protein concentration and purity of the proteins please refer to Chapter 3, section 3.2.2.2)

4.2.2 Sample preparation

Different concentrations of individual whey proteins and their mixtures were prepared in Minimum Essential Medium (MEM). The samples were filter sterilized through low protein binding 0.2 μm Acrodisc® syringe filters (catalogue number 4454, PALL Gelman Ltd, Ann Arbor, MI USA) and kept frozen at –18°C until required.

4.2.3 Cytotoxicity of whey proteins

Semi-confluent HeLa cells grown in 24-well tissue culture plates were incubated with 300 μL of prepared whey protein samples for 60 minutes. Cell morphology was monitored with a microscope (WILD Heerbrugg, Switzerland) and

cell viability was examined using trypan blue dye exclusion method. Details of the procedure are described in Chapter 3 section 3.2.10.

4.2.4 Solubility test

The solubility test method described by Akita and Nakai (1990), with some modification, was used. Briefly, protein solutions were mixed thoroughly in Minimum Essential Medium (MEM) by vortexing for 20 seconds. Aliquots (500 µl) of each protein solution were dispensed in two 2 mL microcentrifuge tubes. One of the tubes was subjected to centrifugation at 15800 x g (Eppendorf 5402 microcentrifuge, Brinkmann Instruments, Mississauga, ON) for 30 min at 5°C. Biuret protein microassay was conducted on non-centrifuged solution and supernatant of centrifuged samples. The Biuret method is described in detail in Chapter 3 section 3.2.6.

4.2.5 *S.* Typhimurium and HeLa cell culture

S. Typhimurium SL1344 culture was used as challenge bacteria for invasion assay. HeLa cells CCL-2 were cultured and maintained in MEM and used between passages 1-20 as described in Chapter 3 sections 3.2.7 and 3.2.8 respectively.

4.2.6 Invasion assay

Invasion assay using mixtures of whey proteins for Random Centroid Optimization involved testing more than 24 samples at a time. Concern about the plate to plate variation prompted the modification of the general protocol to use 96-well plates for invasion assay to fulfill the need for testing several samples at the same time and also decreasing the time and amount of samples and reagents used. To develop a microscale procedure, the surface area of the culturing wells in 24 and 96-well tissue culture plates were measured using a caliper to verify the manufacturer's data and the volume of the required reagents and samples were modified accordingly in order to conduct the tests on a smaller scale. The procedure was tested several times to adjust the experimental conditions for the scaled down protocol. The accuracy of the microscale procedure and significance of difference between the results of invasion assay from the conventional and the modified method were determined by testing the same sample with both methods on the same day. Significance of difference was calculated using paired t-test and no significant difference was observed ($p>0.05$). The results are summarized in Appendix 4.7.1.

4.2.7 Microscale invasion assay

Sterile flat bottom polystyrene 96-well tissue culture plates with lid (Costar® catalogue number 3595, Corning NY) were seeded with 170 µL of suspended HeLa cells, the population density of which was adjusted to 10^5 cells mL^{-1}. As described in more detail in Chapter 3 section 3.2.9, the plates were incubated for 18 hours at 37°C.

S. Typhimurium culture was prepared by transferring a loopful of bacteria from a cultivated LB agar plate to 2 mL of prewarmed LB broth in a borosilicate screw cap tube (20 x 150 mm VWR scientific, Mississauga, ON) and incubated at 37°C overnight. On the following day, the prepared bacterial culture was subcultured at 3:100 (v/v) in pre-warmed (37°C) LB broth and grown for 3 hours at 37°C to obtain the bacteria in exponential phase. Thirty minutes before the bacterial culture was ready, old MEM was removed from the tissue culture plate wells by gently blotting the plate on sterile filter paper pads. Preliminary experiments showed that vacuum suction was not appropriate for the microscale method, since it was too strong for the small size wells and cells would be detached from the well surface which in turn would lead to erroneous results. Monolayers were overlaid with 50 μL of MEM containing the test samples. After incubation for 30 minutes, 5 μL of the 1:6 dilution of bacterial suspension in MEM was introduced into the cultured cells. Cells and bacteria were incubated for 30 minutes to allow bacterial invasion. Extracellular and loosely bound bacteria were removed by washing the cells twice with PBS^{++}. To kill the non-internalized bacteria, cells were incubated for 90 minutes with 85 μL of MEM supplemented with 100 μg mL^{-1} gentamicin (G-1272, Sigma Chemicals, St Louis, MO). HeLa cells were then washed with PBS^{++} to remove antibiotic residues and lysed with 170 μL of filter sterilized 0.1% SDS (w/v) and 1% (v/v) Triton X-100 in PBS^{--}. Released internalized bacteria were serially diluted in PBS^{--} and cultured on LB agar plates by the drop plate method (Facon and Skura, 1996) and incubated for 24 hours at 37°C. Colony forming units (CFU) were determined and compared to the

negative control, which were HeLa cells invaded by *S.* Typhimurium under the same conditions as test the samples but in the absence of whey proteins. Percentage of invasion was calculated and the results were recorded as anti-invasive activity using the following formula:

Percentage of anti-invasive activity = 100-[(CFU $_{\text{protein treated}}$/CFU $_{\text{control}}$) 100]

4.2.8 Random Centroid Optimization (RCO)

Random Centroid Optimization program for Windows designed and developed by Dr. Shuryo Nakai at the University of British Columbia (Vancouver, BC Canada) was used for optimization of the whey protein levels in formulating a mixture with high anti-invasive activity. Program specifications are explained in more detail in Chapter 2 section 2.7.1. Operation chart of the Random Centroid Optimization program is illustrated in Appendix 4.7.2 (a) and (b). The program consists of 3 main steps: random search, centroid search, and mapping.

4.2.8.1 Random search

In random search stage, the components of the mixture, namely whey proteins, which in RCO software are referred to as factors, were defined for the program. Also the concentration range of each factor was assigned as the lower and upper limits for RCO. It was decided not to increase the amount of each whey protein to more than 15

mg mL^{-1} within the whole mixture. Hence, the lower and upper limits for each factor (lactoferrin, α-lactalbumin and β-lactoglobulin) were defined for the program to be 0 and 15 mg mL^{-1} respectively. Random search formulated nine different experimental combinations within the given range for each factor, using a regulated random generator (Details about the regulated random generator are provided in Chapter 2 section 2.7.1). All 9 mixtures were prepared in MEM and subjected to invasion assay. Each mixture was independently tested three times with three replicates each time.

4.2.8.2 Centroid

Results of invasion assays on nine protein mixtures, obtained by random search, were collected. The objective was set to optimize the formulation based on the anti-invasive activity per mg protein used. In other words the goal was to find the best combination of the proteins, which conferred the highest anti-invasive activity with lowest total protein concentration. Responses were calculated as the ratio of anti-invasive activity per mg protein using the following formula:

$[100-(100 \times CFU_{\text{protein treated}}/CFU_{\text{control}})]$/Total Protein concentration

Calculated responses of random search were entered into the RCO program. The centroid search generated 3 more combinations with narrower range. Mixtures were prepared and subjected to invasion assay. Each experiment was conducted three times with three replicates each time.

4.2.8.3 Mapping

After conducting the experiments on combinations generated by the centroid search, the responses were calculated once again as described above and entered into the program as anti-invasive activity per mg protein. All responses for random, centroid search and results of previous invasion assays, presented in section 4.4.4, were combined. The mapping took place to visualize the optimum area and trend. Responses for each factor were mapped individually by entering the factor number and clicking on the mapping option. The flow diagram of Random Centroid Optimization method used in this study is shown in Figure 4.1.

4.2.9 Statistical analysis

All experiments were performed at least in triplicate and in three independent trials. Results represent mean values ± standard deviations unless otherwise stated. Anderson-Darling normality test was done on the data. Significance of the difference between samples and control were evaluated using analysis of variance of logarithmic transformed data by Minitab version 13.32 for Windows (MINITAB™, statistical software Minitab Inc. State College PA USA).

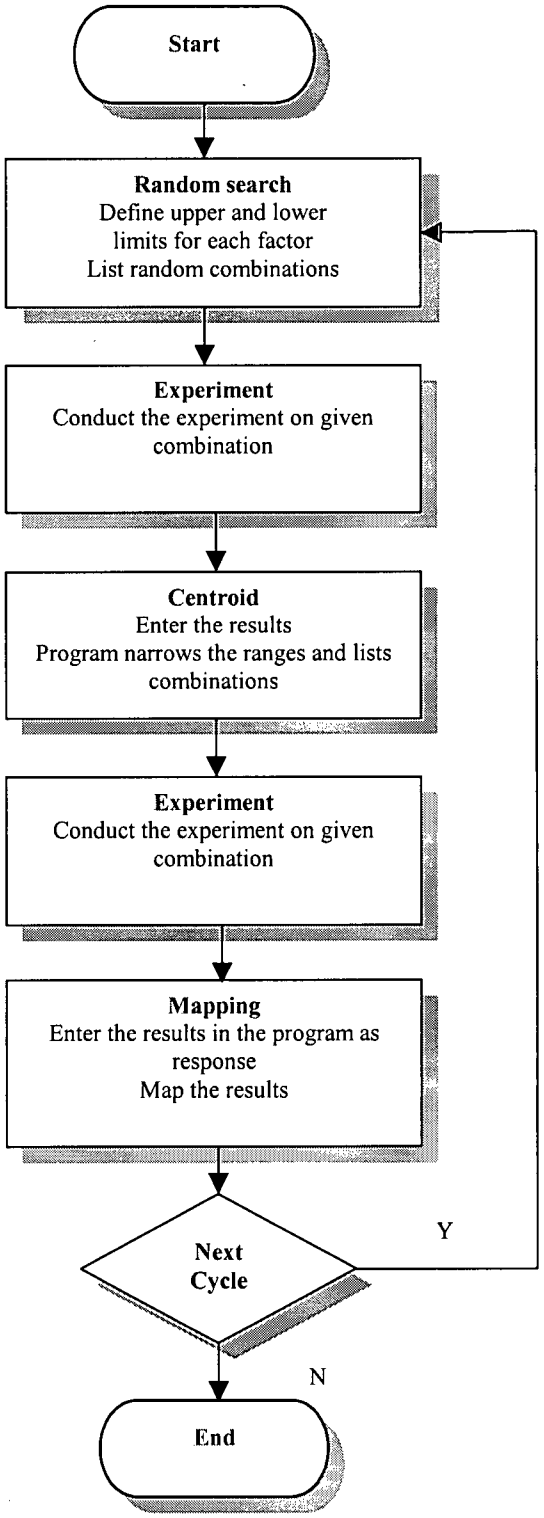

Figure 4.1. Simplified flow diagram of Random Centroid Optimization (RCO) program

4.3 Overview of work plan

4.3.1 Effect of concentration on anti-invasive activity of whey proteins

Our previous studies demonstrated the anti-invasive activity of whey proteins. To determine the effect of concentration on anti-invasive activity of whey proteins and also to identify if the inhibition of invasion follows a trend, a series of experiments were conducted using lactoperoxidase, α-lactalbumin, β-lactoglobulin and lactoferrin. In preliminary experiments, lactoperoxidase at 2, 5 and 10 mg mL^{-1}, was tested for its anti-invasive activity against *S.* Typhimurium. Three of the whey proteins (α-lactalbumin, β-lactoglobulin and lactoferrin) were selected for further experiments and formulation of the mixture. Samples, containing 3, 6, 8 and 15 mg mL^{-1} of each whey protein in MEM, were prepared. Concentrations were selected based on the concentration of whey proteins in the previous study in chapter 3 thus creating a range of lower and higher concentrations as compared to the previous study. Mixtures of the whey proteins with total protein of 12, 24 and 30 mg mL^{-1} were also prepared. Mixtures had equal amounts of each protein. All samples were subjected to HeLa cell invasion assay using *S.* Typhimurium as challenge bacteria.

Experiments were conducted 3 times with 3 replicates each time. Solubility test was conducted to verify the complete solubilization of whey proteins in MEM. HeLa cell viability after exposure to whey proteins was also determined to ensure the whey proteins were not cytotoxic to HeLa cells at the concentrations and duration of exposure used.

4.3.2 Formulating a mixture of whey proteins with optimum anti-invasive activity

Components of a mixture are in close contact with each other. These contacts favour interactions which is concentration dependent. Since these interactions may be additive, antagonistic or synergistic, understanding them is a major factor to consider in any formulation. There are some reports on the synergistic effect of lysozyme and lactoferrin on *E. coli* growth inhibition (Yamauchi, Tomita, Giehl and Ellison, 1993). The effect of interaction among some whey proteins on functional properties such as gelation has been reported earlier (Rojas, Goff, Senaratne, Dalgleish and Flores, 1997). Interaction of lactoferrin with other proteins has been reported. In a study done on bovine whey proteins, researchers found interaction between lactoferrin and β-lactoglobulin (Lampreave, Pineiro, Brock, Castillo, Sanchez and Calvo, 1990). Hekman (1971) also demonstrated the association of lactoferrin and albumin and plasma proteins.

The feasibility of formulating a mixture of whey proteins with optimum anti-invasive activity using Random Centroid Optimization program was investigated in this part of the research.

4.4 Results and Discussion

4.4.1 Solubility test on whey proteins

In investigating the effect of whey proteins on *S.* Typhimurium invasion of HeLa cells, it was necessary to dissolve the whey proteins in MEM to maintain the HeLa cell viability during the experiment. MEM itself is a complex medium containing amino acids, inorganic salts, vitamins and carbohydrate. Since the actual amount of the proteins, which inhibited the invasion of HeLa cells by *S.* Typhimurium, was important in interpreting the results of the invasion assay, it was critical to ensure the complete solubilization of the proteins in MEM. To test the solubility of the whey proteins in MEM, non-centrifuged and supernatant of centrifuged samples were subjected to Biuret protein assay. To eliminate the effect of proteins and phenol red, which are present as normal MEM ingredients, it was decided to use MEM as a negative control. No significant difference ($p>0.05$) between the absorbance of non-centrifuged and supernatant of centrifuged individual whey protein samples was observed (Data not shown). Results suggest the complete solubilization of the whey proteins in MEM at the concentrations used.

4.4.2 Effect of lactoperoxidase concentrations on *Salmonella* Typhimurim invasion of HeLa cells

As cited by Reiter and Härnulv (1984), the reported anti-bacterial activity of lactoperoxidase dates back to observations by Hanssen in 1924. He speculated that an

oxidative enzyme was responsible for the anti-bacterial activity of fresh milk. It was later discovered that presence of two reactants are necessary for anti-bacterial activity of lactoperoxidase. Thus lactoperoxidase together with hydrogen peroxide and thiocyanate was called lactoperoxidase system, which has been extensively studied as a natural anti-bacterial agent in milk.

According to the results presented in chapter 3, while α-lactalbumin, β-lactoglobulin and lactoferrin had substantial anti-invasive activity against *S.* Typhimurium invasion of HeLa cells, lactoperoxidase showed marginal inhibition at the concentrations used. To make a final decision in ruling out or including lactoperoxidase as a potent anti-invasive agent in further studies, a series of experiments were conducted.

Samples containing 2, 5 and 10 mg mL^{-1} lactoperoxidase in MEM were prepared and subjected to HeLa cell invasion assay using *S.* Typhimurium as challenge bacteria. Table 4.1 shows the results of invasion assay on HeLa cells treated with different concentrations of lactoperoxidase. Lactoperoxidase, at the highest concentration (10 mg mL^{-1}), slightly (10%) inhibited *S.* Typhimurium invasion of HeLa cells. Regression analysis (Figure 4.2) showed a significant polynomial relation between the lactoperoxidase concentration and anti-invasive activity with R^2 value of 0.92 (n=9). Considering the low ratio of anti-invasive activity per mg protein and high cost, lactoperoxidase did not seem to be a promising agent for further experiments and it was eliminated from the design. The results also suggest that the mechanism of invasion inhibition is far more complicated than just a general protein-cell membrane

interaction. Otherwise the same concentation of lactoperoxidase and other whey proteins such as lactoferrin would give the same anti-invasive activity and same trend.

Table 4.1. Effect of lactoperoxidase concentration on *Salmonella* Typhimurim invasion of HeLa cells.

Lactoperoxidase concentration (mg mL^{-1})	**Percent anti-invasive activity***
0	0 ± 2.3
2	0 ± 2.9
5	6.7 ± 0.5
10	10 ± 1.4

*Percent anti-invasive = 100 - percentage of invasion
Figures presented are the mean values of the 3 experiments with 3 replicates ± standard deviation.

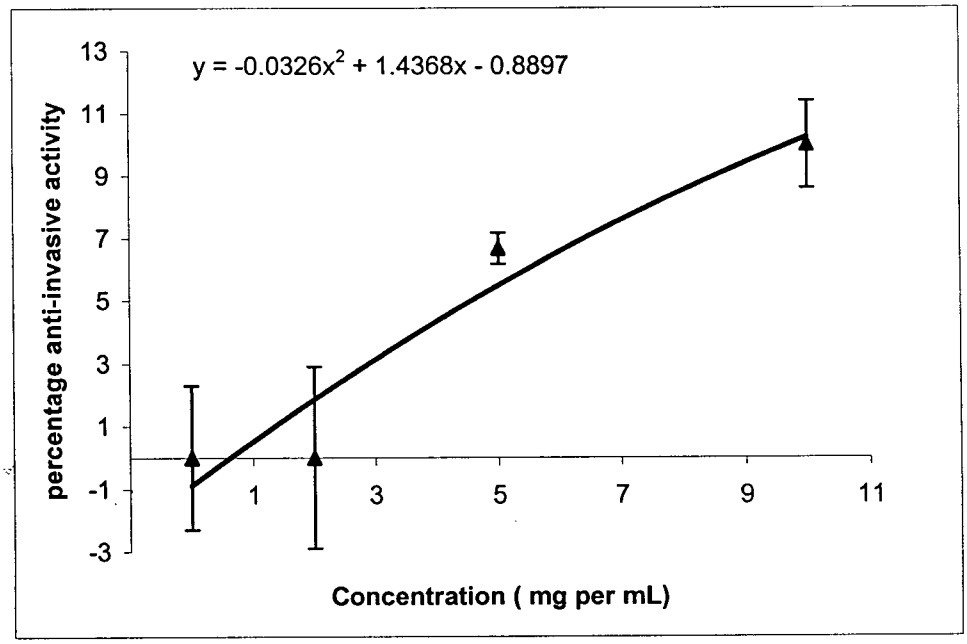

Figure 4.2. Trend line of effect of concentration on anti-invasive activity of lactoperoxidase

4.4.3 Cytotoxicity of whey proteins

Cytotoxicity was a crucial control point in the research and was considered as the gateway to carry on the rest of the experiments. Maintaining the integrity and wholesomeness of the cells is one of the requirements in any experiment using cell culture. Cell morphology and function will change due to cytotoxicity and may cause cell death during the experiment, which in turn may lead to unpredictable and unreliable results. In the present study, it was important to ascertain that the whey proteins had no adverse effect on HeLa cell viability at the concentrations used. Viability of HeLa cells after 60 minutes exposure to whey proteins was tested using trypan blue dye exclusion method and compared to non-treated HeLa cells. The results of cell viability are summarized in Table 4.2. Statistical analysis using paired t-test showed no significant difference ($p>0.05$) between the number of viable cells in non–treated and protein treated samples suggesting that there was no cytotoxicity involved due to presence of the whey proteins at the duration and concentration used for this study. Fetal Bovine Serum (FBS) was also tested for its cytotoxicity along with whey proteins, since it was going to be considered as the second negative control in anti-invasive testing. The FBS level was adjusted to 15 mg mL^{-1} protein in FBS-MEM mixture. No cytotoxicity was observed due to exposure of HeLa cells to FBS.

Table 4.2. Evaluation of cytotoxicity of whey proteins individually and in mixture on HeLa cells

Protein	Standardized number of viable cells relative to control (N x 10^4)
Blank	100
La	104.31 (4.89)
Lg	100.87 (3.02)
Lf	98.90 (7.27)
Mix*	104.95 (9.69)

La: α-lactalbumin, Lg: β-lactoglobulin, Lf: Lactoferrin, The protein content was 15 mg mL^{-1} in MEM.

* Mixture contained 10 mg of lyophilized powder of each individual whey protein (total protein content of 30 mg mL^{-1} prepared in 1 mL of MEM). Figures in brackets are standard deviations

Experiments were conducted in 3 independent trials with 3 replicates. Cells in 8 grid squares were counted for each sample. Paired t-test was performed using MINITAB 13.32.

4.4.4 Effect of α-lactalbumin, β-lactoglobulin and lactoferrin concentration on *S.* Typhimurium invasion of HeLa cells

The objective of this part of the study was to determine if the anti-invasive activity of selected whey proteins was concentration dependent. Although β-lactoglobulin consists of different genetic variants, mostly A and B, it was decided to work with the mixed variant β-lactoglobulin without any further purification for the present part of the study. The reason for this approach was since the final goal of this research was to find a new ingredient for food products formulations, it was more likely that a less purified form would be more desirable for industry from the practical point of view. Hence, it was decided to work with the mixed variant β-lactoglobulin without further purification for the present part of the study.

S. Typhimurium invasion of HeLa cells was significantly inhibited when whey proteins were applied to HeLa cells prior to invasion. This inhibition increased by increasing the whey protein concentration, except for β-lactoglobulin at 15 mg mL^{-1}. The highest inhibition was observed when HeLa cells were pretreated with 15 mg mL^{-1} lactoferrin, which resulted in 78% reduction of *S.* Typhimurium invasion. Statistical analysis showed that the levels of invasion inhibition amongst tested whey proteins were not significantly different ($p>0.05$) at 8-9 mg mL^{-1}. At 15 mg mL^{-1}, lactoferrin had significantly ($p< 0.05$) higher inhibition than the other two whey proteins.

Regression analysis further confirmed the significant relation ($p<0.05$) between whey protein concentrations and their anti-invasive activity. Figure 4.3

shows the trend lines and the results of regression analysis of whey protein concentration and anti-invasive activity. Increasing the concentration of whey proteins up to 9 mg mL^{-1} resulted in higher anti-invasive activity for all whey proteins. Increasing the concentration to 15 mg mL^{-1}, however, changed the regression curve to a polynomial relation for β-lactoglobulin and α-lactalbumin while lactoferrin retained its linear relation with the *S.* Typhimurium invasion inhibition with increasing concentration.

The results proved that whey proteins inhibit the *S.* Typhimurium invasion of HeLa cells in a concentration dependent manner and also suggested that there is a limit for conferring anti-invasive activity by some whey proteins. Anti-invasive activity approached a saturation point by 15 mg mL^{-1} of α-lactalbumin and β-lactoglobulin but lactoferrin continued to progressively impart anti-invasive activity at that concentration.

In a study with human ileostomy glycoprotein as a model for human intestinal mucus, researchers reported a concentration-dependant inhibitory activity of bovine β-lactoglobulin against SfaII fimbriae mediated adhesion of *Escherichia coli.* The optimum inhibition (72%) was observed with β-lactoglobulin at 50 μg mL^{-1}. The same researchers also reported a lower (25% and 19% respectively) anti-adhesion activity for β-lactoglobulin against *S.* Typhimurium and *Salmonella* Enteritidis (Ouwehand, Salminen, Skurnik and Conway, 1997).

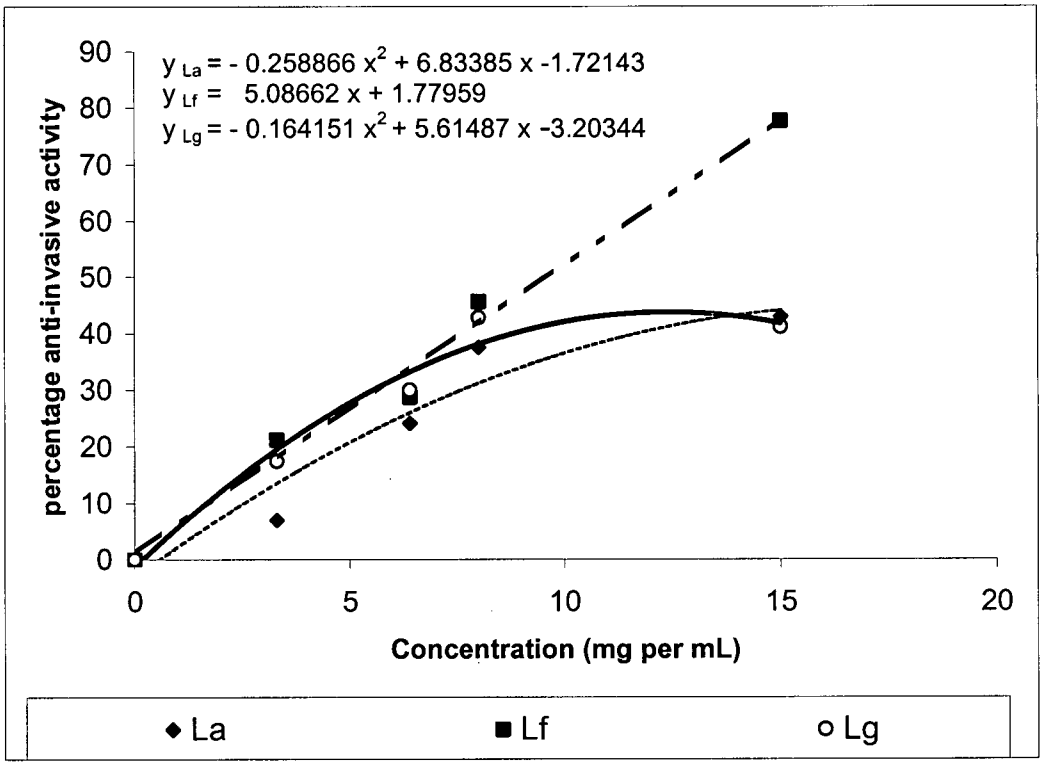

Figure 4.3. Trend lines of effect of concentration on anti-invasive activity of whey proteins. La: α-lactalbumin, Lf: lactoferrin, Lg: β-lactoglobulin (n=9)

Mixtures of lactoferrin, α-lactalbumin and β-lactoglobulin with the total protein concentrations of 9.1, 19.7 and 24.6 mg mL^{-1} showed anti-invasive activity of 9.1, 31.2 and 48% respectively (Figure 4.4). To better understand the effect of whey protein concentration on inhibiting *Salmonella* invasion of HeLa cells relative to protein concentration, anti-invasive activity of each whey protein per mg protein was calculated. Results of anti-invasive activity per mg protein of individual whey proteins and the mixtures are collectively summarized in Table 4.3. Increasing the concentration to 15 mg mL^{-1} did not significantly ($p>0.05$) increase the anti-invasive activity of α-lactalbumin but anti-invasive activity per mg protein decreased from 4.7 to 2.9, which shows the lower efficiency of α-lactalbumin at the higher concentration. β-Lactoglobulin concentrations up to 8.7 mg mL^{-1} also increased the inhibitory effect but increasing the concentration to 15 mg mL^{-1} did not increase the inhibitory effect. The anti-invasive activity per mg β-lactoglobulin decreased from 5.0 at 8.7 mg mL^{-1} to 2.8 at 15 mg mL^{-1}. Lactoferrin, however, showed increasing anti-invasive activity as the concentration increased. The activity per mg lactoferrin did not show a trend and ranged from a low value of 4.5 at 7.9 mg mL^{-1} to a maximum value of 6.6 at 6.3 mg mL^{-1}. A comparison of the results of anti-invasive activity of individual whey proteins and their mixtures showed that while the highest anti-invasive activity achieved for the mixture (24.6 mg mL^{-1}) was 48%, concentrations of 8-9 mg mL^{-1} of each individual whey protein solution of lactoferrin, α-lactalbumin and β-lactoglobulin, respectively had 46, 38 and 43% anti-invasive activity.

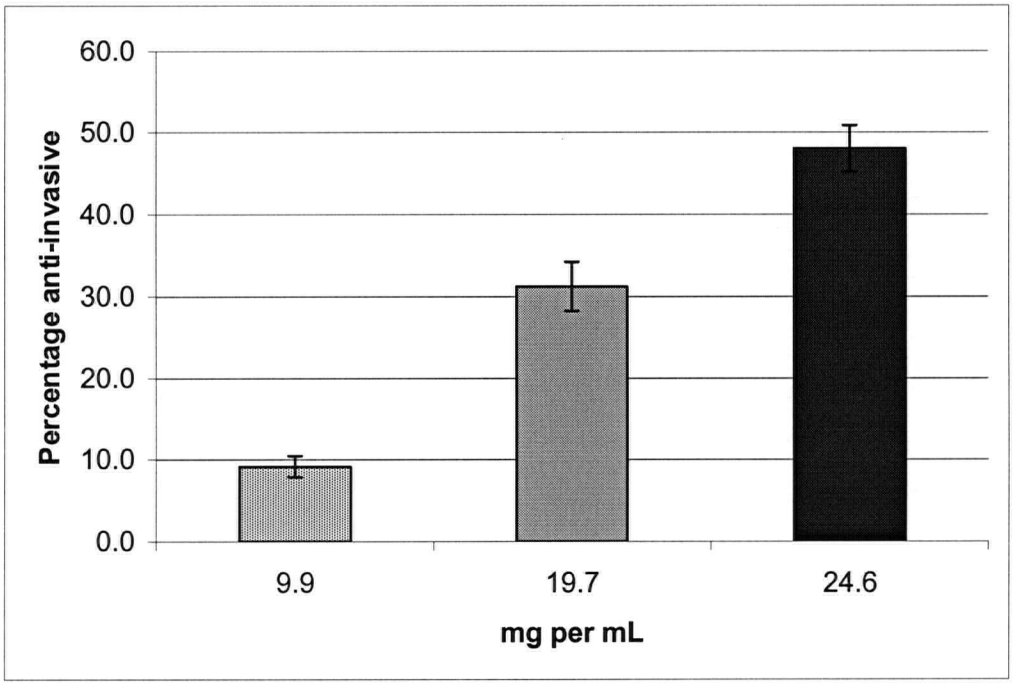

Figure 4.4. Effect of mixture of whey proteins on *S.* Typhimurium invasion of HeLa cells. Data represent the average value obtained from 3 independent experiments done in triplicate. Error bars on each column refer to standard deviations
The numbers on the X axis refer to the concentration of protein (mg mL^{-1}). Each mixture contained equal amounts of lactoferrin, α-lactalbumin and β-lactoglobulin.

Table 4.3. Effect of concentration on anti-invasive activity per mg whey protein

Proteins	Percentage of anti-invasive activity	Anti-invasive activity per mg protein
$La_{3.3}$	6.9	2.1^{abc} (1.7)
$La_{6.4}$	24.0	3.7^{cdef} (1.4)
$La_{8.0}$	37.6	4.7^{fg} (1.0)
La_{15}	42.9	2.9^{bcde} (0.3)
$Lf_{3.2}$	21.1	6.6^{h} (2.2)
$Lf_{6.3}$	28.3	4.5^{efg} (1.3)
$Lf_{7.9}$	46.0	5.8^{gh} (0.9)
Lf_{15}	77.6	5.2^{fgh} (0.2)
$Lg_{3.5}$	17.1	4.9^{fgh} (1.2)
$Lg_{6.9}$	29.7	4.3^{defg} (0.7)
$Lg_{8.7}$	42.9	5.0^{fg} (0.9)
Lg_{15}	41.8	2.8^{bcd} (0.4)
$Mix_{9.9}$	9.1	0.9^{a} (0.2)
$Mix_{19.7}$	31.2	1.6^{ab} (0.4)
$Mix_{24.6}$	48.0	2.0^{ab} (0.2)

La: α-lactalbumin, Lf: lactoferrin, Lg: β-lactoglobulin
Mixture contained equal amount of each individual protein
Figures in brackets are the standard deviations. (n=9)
Subscripts denotes the actual amount of proteins (mg mL^{-1})
Figures with the same superscripts are not significantly different ($p>0.05$)

An interesting conclusion drawn from the results is that, in the mixture of proteins, despite the presence of a significant relationship between individual whey protein concentration and the inhibitory effect, the activity does not seem to be additive, suggesting the possibility of an antagonistic interaction between the components. In other words for the mixture of proteins, we would expect anti-invasive activity at least equal to the sum of individual anti-invasive activities of each protein in the mixture, if no antagonistic effect was involved. Confirming the speculated antagonistic interaction between the whey proteins requires more conclusive investigation. Although the activity per mg protein for the mixture increased with increasing protein concentration, the low activity per mg of protein around 2, for the 24.6 mg mL^{-1} whey protein mixture, can be a limiting factor for further consideration as a potent mixture. The results showed that, while 8 mg mL^{-1} of lactoferrin had a relatively good inhibitory effect (46%) and high activity per mg protein (5.8), lactoferrin at 15 mg mL^{-1} with 78% inhibition and activity per mg of 5.2, has a better potential to be considered as *S.* Typhimurium invasion inhibitor than the other two proteins.

There are only a few reported studies on anti-invasive activity of whey proteins. Antonini *et al.* (1997) reported the reduction of internalized *Listeria monocytogenes* in HT-39 and Caco-2 cell lines in the presence of 1 mg mL^{-1} bovine lactoferrin. Also bovine lactoferrin at 1 mg mL^{-1} was reported to effectively reduce internalization of group A streptococci (Ajello, Greco, Giansanti, Massucci, Antonini and Valenti, 2002). The same researchers also reported the effectiveness of lactoferrin gargle in combination with antibiotic therapy in lowering the number of intracellular

group A streptococci in patients undergoing tonsilectomy. A 50 to 100-fold reduction in the number of internalized *Listeria monocytogenes* in Caco-2, Chang liver cells and primary culture hepatocytes using 1 mg mL^{-1} bovine lactoferrin has also been reported (Ajello, Greco, Donnarumma, Palomba, Polidoro and Valenti, 1998). It has also been shown that lactoferrin at a concentration of 3 mg mL^{-1} was effective in inhibiting the infection of Monkey Embryo Kidney (MEK) cells by Poliovirus Sabin I type I (PV) and also Crandell-Reese Feline Kidney (CRFK) by Feline calcivirus strain 86/68 (FCV) (McCann, Lee, Wan, Roginski and Coventry, 2001). In a study with human lactoferrin and α-lactalbumin, lactoferrin at 300 μg mL^{-1} inhibited adhesion of enteropathogenic *Escherichia coli* (EPEC) to HeLa cells by 24% while no inhibition was observed when α-lactalbumin was used (Araujo and Giugliano, 2001).

4.4.5 Analysis of the whey protein mixtures for their anti-invasive activity

The idea of finding a mixture with improved function has always been behind all formulation technology. The main purpose of any formulation is to identify the optimum level of each component to maximize a certain function or attribute. In all formulation designs, synergism and antagonism are important properties, which require consideration. The previous results showed that α-lactalbumin, β-lactoglobulin and lactoferrin individually possess anti-invasive activity against *S.* Typhimurium.

On the other hand the results of the anti-invasive activity of mixture of whey proteins presented in section 4.4.4 pointed to the possibility of an antagonistic

interaction between lactoferrin, α-lactalbumin and β-lactoglobulin in terms of anti-invasive activity against *S.* Typhimurium invasion of HeLa cells. It has been reported that using the combination of lactoperoxidase and lactoferrin in animal feed had a significant effect. Calves receiving the mixture had healthier vili and lower *Escherichia coli* counts in the jejunum (Van Leeuwen, Huisman, Kerkhof, Kussendrager and Van Leeuwen, 1998). In another study, *E. coli* infected calves receiving a lactoperoxidase and lactoferrin mixture had faster recovery and lacked the hypothermia and clinical depression compared to the control group (van Hooijdonk., Kuussendrager and Steijns, 2000). The structure of lactoferrin allows interaction with other macromolecules (Brock, 1997). The interaction of lactoferrin and β-lactoglobulin has also been reported (Lampreave *et al.*, 1990).

In this part of the research, Random Centroid Optimization was employed with the idea of extending the anti-invasive activity of whey proteins by formulating a mixture with high anti-invasive activity and also to determine if the proteins have synergistic or antagonistic activity in inhibiting the invasion of HeLa cells by *S.* Typhimurium. Random search produced 9 mixtures within the defined range of 1-10 mg mL^{-1} for each whey protein. Mixtures were prepared and tested for their anti-invasive activity. Results are summarized in Table 4.4.

The highest anti-invasive activity from the random search was 39.6%, which corresponds to mixture 3 with a total protein concentration of 11.31 mg mL^{-1}. Considering the anti-invasive activity per mg of protein, mixture 1 with 5.55 mg mL^{-1}

protein and 30.2% anti-invasive activity had the best anti-invasive to protein ratio of 5.44.

Since the goal of this part of the study was to formulate a mixture with improved anti-invasive properties with the minimum amount of protein, the formulation was optimized based on the mg protein used. Therefore results of anti-invasive activity per mg protein were calculated and used as response in the centroid part of the program. The program analyzed responses and 3 more combinations with narrower range were generated and subjected to HeLa cell invasion assay.

Mixture number 11 showed 32% anti-invasive activity with highest anti-invasive to protein ratio (4.14) amongst the last 3 combinations. Despite a slightly better anti-invasive activity compared to mixture 1, the efficiency of the mixture was slightly lower, since more protein was involved in providing the anti-invasive activity. The results presented in Table 4.4, however, did not clearly show the types of interactions, if any among the whey proteins. This was further analysed in regression analysis and the mapping section.

The expected individual anti-invasive activity of each whey protein was calculated using the regression equations for lactoferrrin, α-lactalbumin and β-lactoglobulin (Figure 4.3). The predicted value of anti-invasive activity of the mixture of the proteins, assuming that the activity is additive, was also calculated and compared to the observed anti-invasive activity of the mixtures (Table 4.5). The results show that at lower total protein concentrations, such as mixtures 1 and 9, the anti-invasive activity seemed to be additive, especially when the concentration of

lactoferrin was also low. Increasing the total protein resutled in a larger difference between the predicted and observed values for anti-invasive activity. Mixtures 4, 6 and 7 had the highest total protein content and also had the highest difference between the predicted and observed anti-invasive activity. It is speculated that the total protein concentration and also the concentration of the lactoferrin in the mixture are important in the type of the interactions that occurred amongst these whey protins in conferring anti-invasive activity. At lower concentrations, such as in mixture 1, the interaction was additive. Increasing the total protein concentration changed this interaction to antagonistic. More research needs to be done to investigate the effect of protein-protein interactions on anti-invasive activity of whey proteins.

Table 4.4. Combination of the proteins generated by random and centroid search and responses after conducting the HeLa cell invasion assay using *S.* Typhimurium as challenge bacteria

Cycle 1 **Random**	**La** mg mL^{-1}	**Lg** mg mL^{-1}	**Lf** mg mL^{-1}	**Total protein** mg mL^{-1}	**Percentage of Anti-invasive[a] activity**	**Anti- invasive activity per mg protein[a]**
MIX1	1.620	1.370	2.564	5.554	30.2 (2.94)	5.44 (0.53)
MIX2	3.392	3.857	7.902	15.152	37.4 (1.96)	2.47 (2.66)
MIX3	2.565	3.623	5.127	11.315	39.6 (3.56)	3.50 (2.34)
MIX4	7.356	6.208	5.753	19.317	24.5 (3.26)	1.27 (2.11)
MIX5	1.765	7.226	1.050	10.042	22.6 (2.28)	2.25 (0.97)
MIX6	4.057	7.327	5.100	16.484	7.20 (2.45)	0.43 (0.67)
MIX7	7.605	5.540	5.426	18.572	35.1 (0.20)	1.89 (2.55)
MIX8	2.413	1.007	7.794	11.214	36.8 (5.80)	3.29 (1.37)
MIX 9	1.813	3.623	1.608	7.045	25.3 (2.98)	3.59 (0.61)
Centroid						
MIX10	2.003	2.873	3.104	7.980	5.40 (0.82)	0.68 (0.10)
MIX11	1.955	2.006	3.991	7.953	32.9 (1.05)	4.14 (0.13)
MIX12	2.197	2.007	5.163	9.368	8.2 (1.21)	0.88 (0.15)

[a] each value represents the mean value of triplicates from three independent invasion assays. Figures in brackets corresponds to standard deviation.

Table 4.5. Comparison of the expected anti-invasive activity and the observed anti-invasive activity of the whey proteins

	Predicted percentage of anti-invasive activity[1]				*Observed % anti-invasive activity*
	La[2]	***Lg***[2]	***Lf***[2]	***Sum***	
MIX1	8.7	4.2	14.8	27.7	30.2 (2.94)
MIX2	18.5	16.0	42.0	76.5	37.4 (1.96)
MIX3	14.1	15.0	27.9	56.9	39.6 (3.56)
MIX4	34.5	25.3	31.0	90.9	24.5 (3.26)
MIX5	9.5	28.8	7.1	45.5	22.6 (2.28)
MIX6	21.7	29.1	27.7	78.6	7.20 (2.45)
MIX7	35.3	22.9	29.4	87.5	35.1 (0.20)
MIX8	13.3	2.3	41.4	57.0	36.8 (5.80)
MIX 9	9.8	15.0	10.0	34.8	25.3 (2.98)
Mix 10	10.9	11.6	17.6	40.1	5.40 (0.82)
Mix 11	10.7	7.4	22.1	40.1	32.9 (1.05)
Mix 12	12.0	7.4	28.0	47.5	8.2 (1.21)

[1] Predicted percentage anti-invasive activity for each protein was calculated using the regression formula presented in Figure 4.3

[2] La: α-lactalbumin, Lf: lactoferrin, Lg: β-lactoglobulin

Figures in brackets correspond to the standard deviation.

Results of invasion assay from random, centroid and previous results summarized in Table 4.3 were combined and used in mapping. The computer program provided an optimum level along with a map of trend lines pointing towards the possible optimum for each protein. The provided optimum level is not necessarily the global optimum. Underneath each map there is an X co-ordinate of the best point, which can be shifted to left and right manually using a scroll box provided under each map (for the location of scroll box please refer to Figure 4.5).

Trend lines change wheh the co-ordinate is moved. In the mapping section, the co-ordinate was moved until better trend lines were achieved. The more trend lines pointing to a certain area, the higher the possibility of the global optimum residing in that area (Nakai, Dou, Lo, and Scaman, 1998). The scroll box movement was conducted based upon the following rule:

"If the trend lines point in the opposite direction to the scroll box's movement from the current best point, mapping should be repeated by moving the scroll box in the other direction until the directions of both the box movement and trend lines match" (Nakai *et al.*, 1998).

Maps for each factor with better trendlines, as described by the above rule, were selected (Figure 4.5.). In the mapping section, X and Y axes of the map respectively refer to mg protein and anti-invasive activity per mg protein. The trend lines are directed toward the optimum. The results suggest that the optimum combination will be achieved when low levels of α-lactalbumin and β-lactoglobulin and high level of lactoferrin are used suggesting that the anti-invasive activity of

lactoferrin is negatively affected by α-lactalbumin and β-lactoglobulin. Decreasing the level of these two proteins may minimize the antagonistic effect. Since the defined range for whey proteins was 0 to 15 mg mL^{-1}, mathematical explanations would be if lactoferrin approaches the higher limit (15 mg mL^{-1}) and α-lactalbumin and β-lactoglobulin approach the lower limit (0 mg mL^{-1}), we would have highest anti-invasive activity per mg protein used. The finding suggests that by using lactoferrin individually rather than in the mixture, a higher anti-invasive per mg protein will be achieved. Ouwehand *et al.* (1997) found reduced adhesion of *S.* Typhimurium to human ileostomy glycoprotein in the presence of β-lactoglobulin, which was not in agreement with their previous finding where β-lactoglobulin rich fraction was used. They attributed the difference of results to the presence of either lactose or α-lactalbumin as contaminant in their fraction. They suggested that lactose or α-lactalbumin may interact with β-lactoglobulin and abolish its anti-adhesive activity (Ouwehand, *et al.*, 1997).

The result of this part of the present study suggests that, at this point, there is not sufficient data to support the feasibility of formulating a mixture of whey proteins, namely lactoferrin, α-lactalbumin and β-lactoglobulin, with high anti-invasive efficiency due to the antagonistic interaction among the proteins. Lampreave *et al.* (1990) reported interaction of bovine lactoferrin with β-lactoglobulin and α-albumin but no interaction between lactoferrin and α-lactalbumin was found in the current study. The results suggest a need for further investigation of the mechanism of whey protein interactions and their effect on anti-invasive activity.

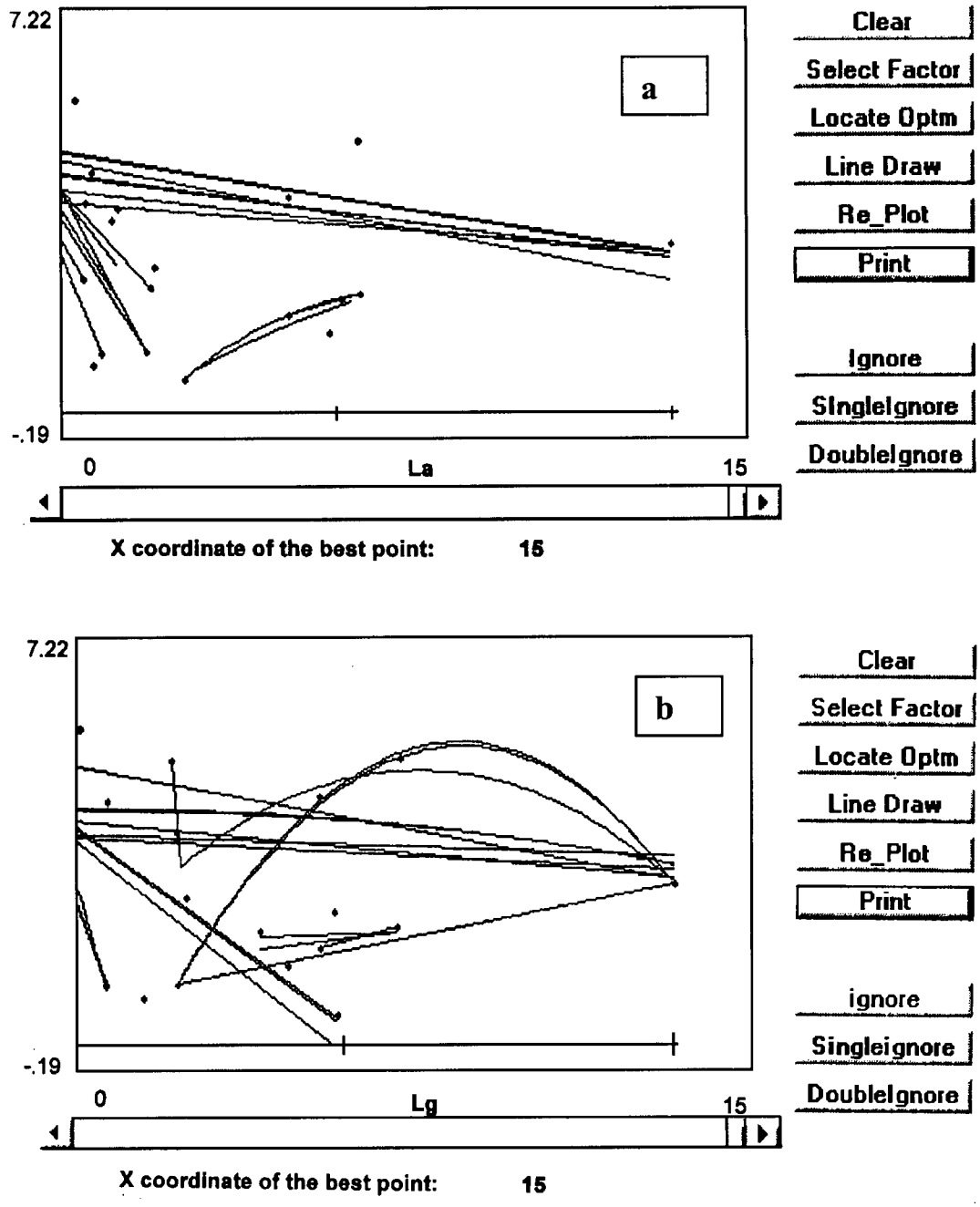

Figure 4.5. See next page for legend

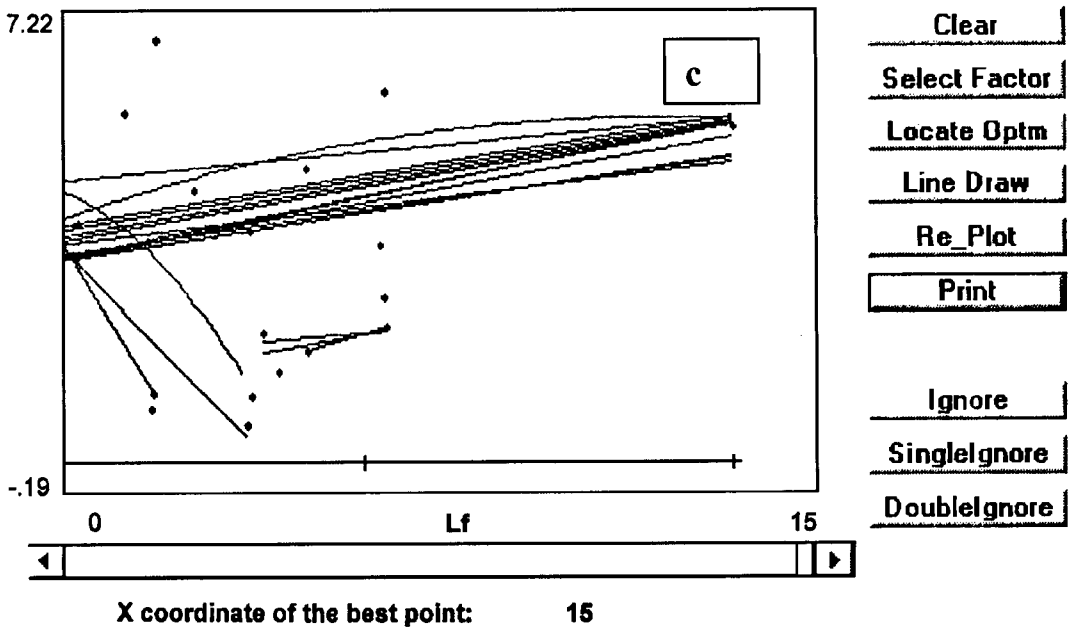

Figure 4.5. Selected maps from the Random Centroid Optimization
a) La: α-lactalbumin, b) Lg: β-lactoglobulin and c) Lf: lactoferrin. X and Y axes respectively correspond to: mg mL^{-1} of protein and anti-invasive activity per mg protein. Maps were prepared by combining data from Tables 4.3 and 4.4.

4.5 Conclusion

Whey proteins are capable of inhibiting the *S.* Typhimurium invasion of HeLa cells at concentrations that were not cytotoxic. Regression analysis showed a significant ($p<0.05$) relation between the tested whey protein concentrations and anti-invasive activity. Also the results proved that despite the difference in trend, the inhibition was concentration dependant for all tested whey proteins. Lactoperoxidase, however had less anti-invasive activity per mg of protein against *S.* Typhimurium than the other whey proteins tested. Another conclusion which can be drawn from the results with lactoperoxidase is that the observed anti-invasive activity with other whey proteins is not a simple protein-bacteria interaction and the mechanism is more complicated.

The use of RCO to study the feasibility of formulating a mixture of proteins with anti-invasive activity is reported for the first time. Results of RCO suggested an antagonistic effect of lactoferrin, α-lactalbumin and β-lactoglobulin mixtures in conferring anti-invasive activity against *S.* Typhimurium invasion of HeLa cells.

There is a need for more research on the mechanism by which whey proteins inhibit invasion of HeLa cells by *S.* Typhimurium.

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4.7 Appendix

4.7.1 Comparison of original and microscale invasion assay

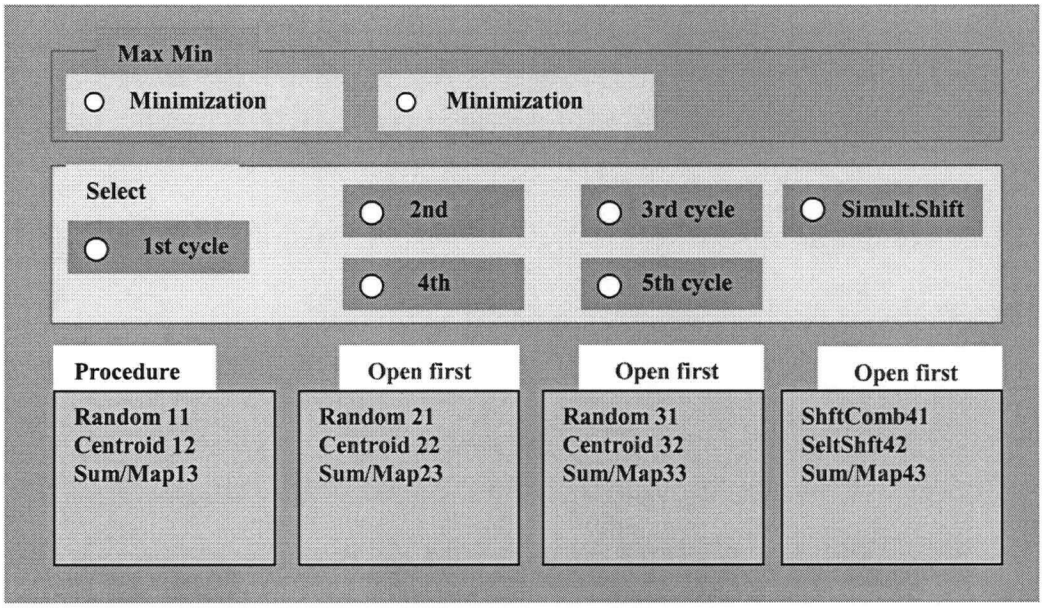
Number of CFU of internalized *S.* Typhimurium recovered from HeLa cells

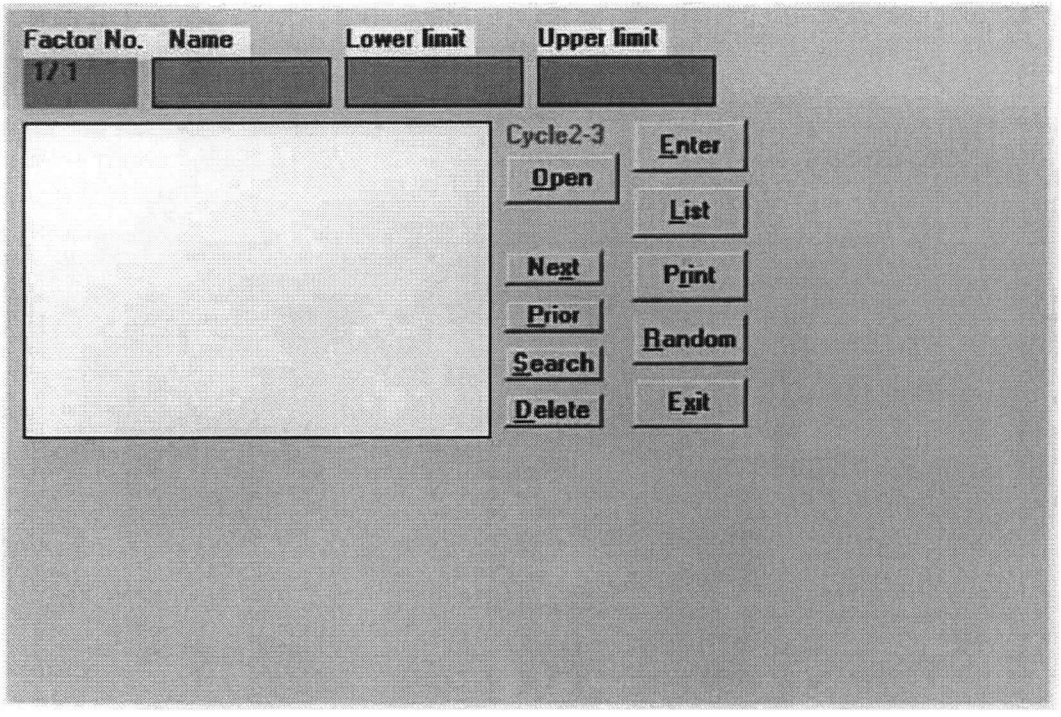
	CFU x 10^2	Standard Deviation
96 well	33.2	6.06
24 well	38.3	5.58

Number of replicates in each experiment (n=6 for 24 well plate, n=12 for 96 well plate)

t-test was conducted using Minitab 13.32 (p>0.05)

4.7.2 Operation Charts of Random Centroid Optimization program





CHAPTER FIVE

Effect of whey proteins on *Salmonella* Typhimurium growth, aggregation and invasion

5.1 Introduction

There are always two approaches for finding any remedy for fighting disease. One approach is to design a substance or procedure, which specifically interferes with the process of disease advancement and settlement. This approach is the core idea behind most pharmaceutical research for drug development. The other approach, "non-targeted screening", is to find practices or compound (s), either synthesised or natural, and test their effectiveness towards a specific health related complication (Ansel, Popovich and Allen, 1995). For non-targeted screening, once the remedy is found effective, understanding the mechanism by which it prevents the development of disease plays an important role in further studies and improving the efficiency of the newly found therapy.

In the past two decades, attempts were made to understand the natural and inherent immune systems by which animals and plants combat microbial disease. The efforts resulted in discovery of a vast number of natural antimicrobial peptides both in animals and plants (Pellegrini, Dettling, Thomas and Hunziker, 2001).

Human and bovine milk have been considered one of the earliest disease fighting substances. For centuries, scientists have been trying to solve the mystery of the aforementioned property of this early human food and the quest still continues. Several milk proteins and their peptides are reported to have anti-bacterial activity (Naidu 2002; Bostwick, Steijns and Braun, 2002).

The invasion inhibition property of some whey proteins was reported in previous chapters of the current study. Lactoferrin, α-lactalbumin and β-lactoglobulin

were found effective in inhibiting *S.* Typhimurium invasion of HeLa cells. For decades the anti-bacterial effect of lactoferrin, either bactericidal or bacteriostatic, was attributed to its iron-binding properties and all evidence pointed to the iron-dependant mechanisms. More recently, iron-independent bactericidal activities have been reported for lactoferrin (Naidu and Arnold, 1997). Numerous mechanisms were proposed for anti-bacterial activity of lactoferrin and its peptides including binding to bacteria and damaging the bacterial outer membrane (Ellison and Giehl, 1991). Although most of the antimicrobial studies on whey proteins focus on lactoferrin and its peptides, recently, β-lactoglobulin and α-lactalbumin have caught the attention of researchers. At the time of writing this manuscript, limited information was available on antimicrobial properties of β-lactoglobulin and α-lactalbumin and there was no information on the anti-invasive activity or the mechanism of bacterial growth inhibition of β-lactoglobulin or α-lactalbumin in scientific literature databases. Although, there has been some research on anti-bacterial activity of whey protein hydrolysate and more specifically active peptides (Branen and Davidson, 2000), utilization of a less purified form is more desirable for industrial application due to the high cost of purification procedures.

This part of the research was initiated with the main objective of better understanding the underlying mechanism by which whey proteins inhibit *S.* Typhimurium invasion of HeLa cells. Although achieving more in-depth information requires specific investigations at the molecular and cellular level, our goal was to provide some evidence, which could serve as preliminary studies and ultimately to

narrow down future research avenues for studying the mechanism of invasion inhibition.

5.2 Materials and methods

5.2.1 Whey proteins

Bovine whey proteins, lactoferrin (L4765), α-lactalbumin Type III (L6010) β–lactoglobulin 3X crystallized (L0130) β-Lactoglobulin A (Sigma L7880), β-lactoglobulin B (Sigma L8005) and lactoperoxidase (L2005) were purchased from Sigma Chemicals (St Louis, MO. USA). Protein purity was assessed by SDS-PAGE. Protein concentration was measured by UV absorbance of a 1% solution at 280 nm and using extinction coefficient of the individual whey proteins. For details please refer to Chapter 3, section 3.2.2.2.

5.2.2 Whey protein sample preparation

Concentrations of lactoferrin, α-lactalbumin and β-lactoglobulin in LB broth were prepared and filter sterilized using low protein binding 0.2 μm Acrodisc® syringe filters (catalogue number 4454, PALL Gelman Ltd, Ann Arbor, MI USA).

5.2.3 Bacterial culture

S. Typhimurium SL1344 culture was maintained on LB agar plates at 4°C for up to 4 weeks and used as challenge bacteria for invasion assays and growth inhibition assays. Details of the procedure can be found in Chapter 3, section 3.2.7.

5.2.4 HeLa cell culture

HeLa cells CCL-2 were cultured and maintained in Minimum Essential Medium (MEM) and used between passages 1-20. The details of the methods are described in Chapter 3, section 3.2.8.

5.2.5 Anti-bacterial assay

A loopful of *S.* Typhimurium SL 1344 was taken from the cultured LB agar plate into 2 mL of LB broth in borosilicate screw cap culture tubes (20 x 150 mm VWR Scientific, Mississauga, ON) and incubated at 37°C. After 16 hours incubation, 300 μL of the bacterial suspension was transferred to 10 mL of fresh, pre-warmed (37°C) LB broth in borosilicate screw cap culture tubes and incubated for 3 hours at 37°C. The latter incubation produced a exponential phase culture in which *S.* Typhimurium are more active and invasive. Prepared whey protein samples in LB broth (1200 μL) were warmed to 37°C and inoculated with 20 μL of prepared bacterial suspension, to yield the same bacteria: protein concentration ratio as in the invasion assays. The population density of bacteria was around 10^7 CFU per mL. LB

broth provides a good source of nutrients for the growth of *Salmonella* as apposed to MEM. Aliquots (50 μL) were taken at 0, 4, 16 and 24 hours. Serial dilutions were made and cultured on LB agar plates using drop plate method. Colony forming units were counted after 24 hours incubation at 37°C and compared to the LB broth samples inoculated with bacterial culture in the absence of whey protein, which served as negative control. A sample containing ethanol 75% (v/v) instead of test protein was prepared and used as positive control. Assays were conducted in three independent experiments with duplicate measurements.

5.2.6 Bacterial aggregation

To determine if bacterial clumping was responsible for the observed lower invasion in samples containing the whey proteins, bacterial aggregation test was performed. Whey protein samples and *S.* Typhimurium culture were prepared as described in sections 5.2.2 and 5.2.3 respectively. Whey protein samples (1200 μL) were inoculated with 20 μL of exponential phase bacterial culture and incubated at 37°C. Samples were taken at 0, 4, 16 and 24 hours for microscopy. An 1-2 μL aliquot of the whey protein treated bacterial suspension was placed on a microscope slide and covered by a cover slip. Cover slip edges were sealed with clear nail polish to avoid moisture loss. Aggregation was monitored qualitatively, using a phase contrast microscope (Zeiss Axioskop). Pictures were taken using a X100 Plan Neofluar objective lens and using differential interference contrast (DIC) microscopy (Zeiss Axioplan2 microscope – DVC camera and Northern Eclipse V. 6.0 imaging

program). For each slide, the whole area under the coverslip was scanned to monitor aggregate formation. A positive aggregation control was prepared using *S.* Typhimurium LPS antibody developed in rabbit (Difco 2948-47-6 Detroit, Michigan).

5.2.7 Pre-incubation of HeLa cells with whey proteins prior to invasion

The original assay procedure was modified to determine the effect of pre-incubation of HeLa cells with whey proteins prior to invasion. HeLa cells were grown to confluency and trypsinized. The population density of the cells was adjusted to 10^5 cells mL^{-1}. Sterile flat bottom polystyrene 96-well tissue culture plates with lid (Costar® catalogue number 3595, Cornining NY) were seeded with 170 µL of suspended HeLa cells. Plates were incubated for 18 hours at 37°C in 5% CO_2, 95% air incubator. *S.* Typhimurium culture was prepared by transferring a loopful of bacteria from LB agar cultured plate (see section 3.2.7.1 for more details) to 2 mL of LB broth prewarmed to 37°C, in a borosilicate screw cap tube (20 x 150 mm VWR scientific, Mississauga, ON) and incubated at 37°C overnight. On the following day, the bacterial culture was subcultured at 3:100 (v/v) in pre-warmed (37°C) LB broth and grown for 3 hours at 37°C to obtain bacteria in exponential phase. Thirty minutes before the bacterial culture was ready, old MEM was removed from the tissue culture plate wells by gentle blotting of the plate on sterile filter paper pads. Monolayers were overlaid with 50 µL of MEM containing whey protein samples. After incubation for 30 minutes, HeLa cells were washed 3 times with PBS^{++}. HeLa cells were

supplemented with 50 μL of MEM and 5 μL of the 1:6 dilution of bacterial suspension in MEM. Cells and bacteria were incubated for 30 minutes to allow bacterial invasion. Extracellular and loosely bound bacteria were removed by washing the cells twice with PBS^{++}. To kill the non-internalized bacteria, cells were incubated for 90 minutes with 85 μL of MEM supplemented with 100 μg mL^{-1} gentamicin (G-1272, SIGMA Chemicals, St Louis, MO). HeLa cells were then washed with PBS^{++} to remove any antibiotic residues and lysed with 170 μL of filter sterilized 0.1% SDS (w/v) and 1% (v/v) Triton in PBS^{--}. Released internalized bacteria were serially diluted in PBS^{--} and cultured on LB agar plates by the drop plate method and incubated for 24 hours. Bacterial colony forming units (CFU) were determined and compared to the negative control which were HeLa cells invaded by *S.* Typhimurium under the same condition as the test samples but in absence of whey proteins. Percentage of invasion was calculated and the results were recorded as anti-invasive activity using the following formula:

Percent anti-invasive activity = $100-[(CFU_{\text{protein treated}}/CFU_{\text{control}}) \times 100]$

5.2.8 Pre-treating of *S.* Typhimurium with whey proteins prior to invasion

A set of experiments was conducted to investigate the effect of pre-incubation of *Salmonella* with whey protein prior to the invasion assay. HeLa cells and bacterial culture were prepared as previously detailed. Briefly, to each well of a 96 well tissue culture plate, 170 μL of the HeLa cell suspension with the population density of 10^5 cells mL^{-1} was added and incubated for 18 hours. A loopful of *S.* Typhimurium

culture was transferred to 2 mL of LB broth and incubated for 16 hours. After diluting the bacterial suspension in LB broth 3:100 (v/v) and incubating for 3 hours, the bacterial culture was further diluted in MEM in 1:6 ratio. Protein solutions were prepared in MEM followed by filter sterilization. Prior to introducing to the HeLa cells, 500 μL of each protein solution was incubated with 50 μL of *S.* Typhimurium culture in MEM for 30 minutes at 37°C, 5% CO_2, 95% air. Fifty five μL aliquots of protein-*Salmonella* suspension were introduced to each well followed by the incubation at 37°C, 5% CO_2, 95% air for 30 minutes HeLa cells were then washed twice with PBS^{++} and incubated for 90 minutes with 85 μL of MEM supplemented with 100 μg mL^{-1} gentamicin. HeLa cells were then washed with PBS^{++} and lysed with 170 μL of filter sterilized 0.1% SDS (w/v) and 1% (v/v) Triton in PBS^{--}. Colony forming units of internalized bacteria were determined using drop plate method. A simplified flow diagram of the procedures is illustrated in Figure 5.1.

5.2.9 Statistical analysis

All experiments were performed in triplicate and in three independent trials, unless otherwise stated. Results are represented as mean values ± standard deviations. Data were subjected to the Anderson-Darling normality test. Significance of the difference between samples and control were evaluated by analysis of variance of logarithmic transformed data using Minitab version 13.32 for Windows (MINITAB™, statistical software Minitab Inc. State College PA USA).

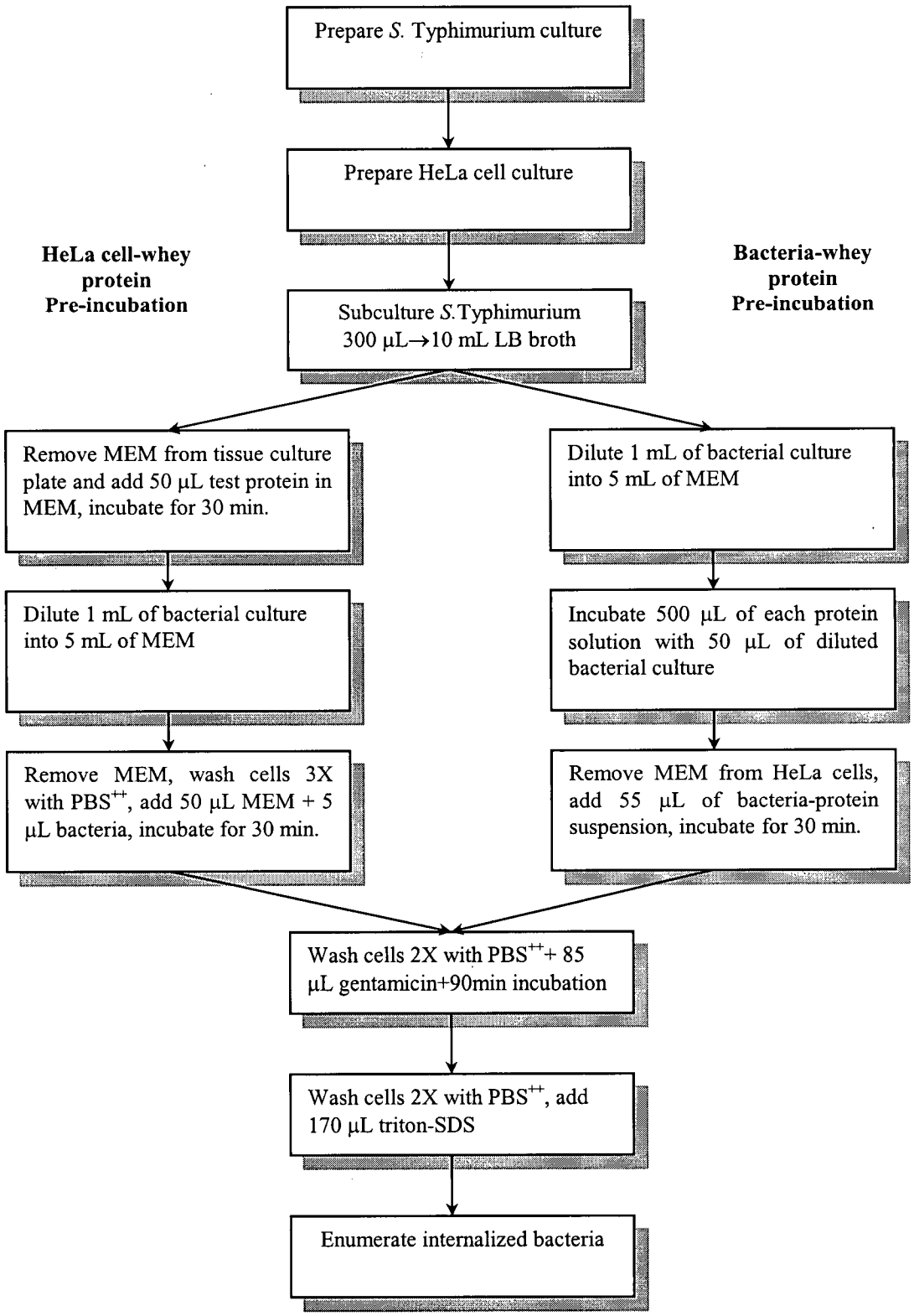


Figure 5.1. Flow diagram of the invasion assay procedures for HeLa cell-whey protein or *S.* Typhimurium-whey protein pre-incubation

5.3 Overview of work plan

Results of the previous studies revealed the effectiveness of using whey proteins to inhibit the *S.* Typhimurium invasion of HeLa cells, as evidenced by the lower CFU recovered from whey protein treated cells compared to negative control. Growth inhibition of *S.* Typhimurium in the presence of whey proteins was studied to further investigate the cause of the observed lower CFU. Since aggregation of bacteria also could result in lower CFU counts, effect of whey protein on clumping behaviour of *S.* Typhimurium was investigated.

In this part lactoferrin, α-lactalbumin and β-lactoglobulin at 10 and 15 mg mL^{-1} were evaluated for their bactericidal or bacteriostatic and bacterial aggregation formation properties. Moreover, experiments were conducted by preincubating, 10 mg mL^{-1} of whey proteins with either *S.* Typhimurium or HeLa cells separately before the invasion assay to elucidate the effect of these procedural changes on the anti-invasive activity of the proteins. To have a better understanding of the inhibition mechanism and also to make the comparison more conclusive the ratio of bacteria, HeLa cells and whey protein concentration was kept consistent with the previous studies. Lastly, based on the results of this chapter, a mechanism by which whey proteins may inhibit *S.* Typhimurium invasion of HeLa cells will be discussed.

5.4 Results and discussion

5.4.1 Anti-bacterial activity of whey proteins

To extend the investigation on confirming or ruling out the possible mechanism by which whey proteins inhibit the *S.* Typhimurium invasion of HeLa cells, a series of studies, starting with the bacterial growth inhibition experiment, was conducted. Exponential phase *S.* Typhimurium cultures were incubated with 10 and 15 mg mL^{-1} of whey proteins. β-lactoglobulin variants A and B were included in this investigation to determine if the structural difference has any effect on the anti-bacterial activity. None of the tested whey proteins inhibited the growth of *S.* Typhimurium during 24 hours of incubation at 10 mg mL^{-1} (Figures 5.2 and 5.3). Results showed that increasing the concentration of the whey proteins to 15 mg mL^{-1} did not inhibit the growth of *S.* Typhimurium (Figure 5.4).

Human lactoferrin (2 mg mL^{-1}) was reported to have bacteriostatic activity against *S.* Typhimurium SL696 (Ellison and Giehl, 1991). The activity was enhanced to bactericidal when lysozyme was used in combination with human lactoferrin. The authors attributed this synergistic effect to lactoferrin damaging the bacterial outer membrane and providing the opportunity for lysozyme to act. Researchers reported a significant decrease in the number of viable *Helicobacter pylori* using 2 mg mL^{-1} bovine lactoferrin after at least 2 days of incubation at 37°C. The inhibition effect was not significantly different when 2 mg mL^{-1} of lysozyme was used in combination with lactoferrin. Interestingly the authors reported that lactoferricin B was not effective in inhibiting growth of *Helicobacter pylori*. They ascribed the inhibition to the iron-

binding property of lactoferrin and also acknowledged the contribution of other unknown mechanisms (Dial, Hall, Serna, Romero, Fox, and Lichtenberger, 1998). Studies on anti-bacterial activity of whey proteins namely lactoferrin show conflicting results (Rainard, 1986). This can be the result of different procedures used for the assays. For example it was demonstrated that the bacterial population at the start point is an important factor in effectiveness of the anti-bacterial substance. Anti-bacterial activity for lysozyme and lactoferrin combination decreased when the inoculum size was increased from 5×10^5 to 5×10^7 CFU per mL (Ellison and Giehl, 1991). Variability of the results reported on anti-bacterial activity of whey proteins can be attributed to different procedures used in various studies.

Results of this part of the study clearly show that whey proteins were neither bactericidal nor bacteriostatic at the concentrations and conditions used. Therefore bactericidal activity was not responsible for the observed lower CFUs recovered from invasion assays in the previous studies. The finding suggested that there are other mechanisms involved in the inhibition of *S.* Typhimurium invasion of HeLa cells. This is in agreement with the results of Antonini and co workers (1997) who found that apo-lactoferrin and iron and manganese saturated lactoferrin at 8 and 4 mg mL^{-1} had a weak anti-bacterial activity against *Listeria monocytogenes* while those proteins at 1 mg mL^{-1} were all significantly effective in inhibiting invasion of HT-29 and CaCo-2 cells by *Listeria monocytogenes*. Other researchers also found that although 300 μg mL^{-1} of human lactoferrin was effective in preventing the adherence of enteropathogenic *E. coli* (EPEC) to HeLa cells, the protein had no bactericidal or

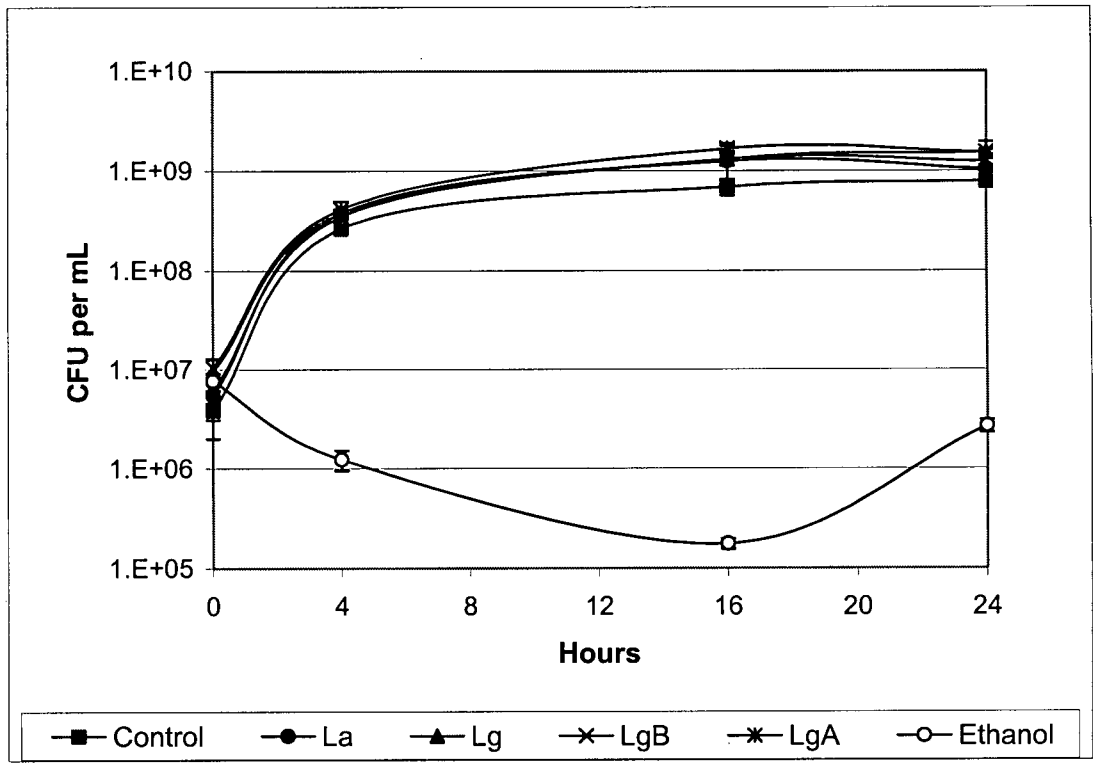


Figure 5.2. Effect of 10 mg mL^{-1} α-lactalbumin (La), β-lactoglobulin (Lg), β-lactoglobulin A (LgA), β-lactoglobulin B (LgB) on *S.* Typhimurium survival in LB broth during 24 hours of exposure at 37°C. Counts obtained by plating on LB agar. The results are the mean value of three experiments. Bars represent standard deviation.

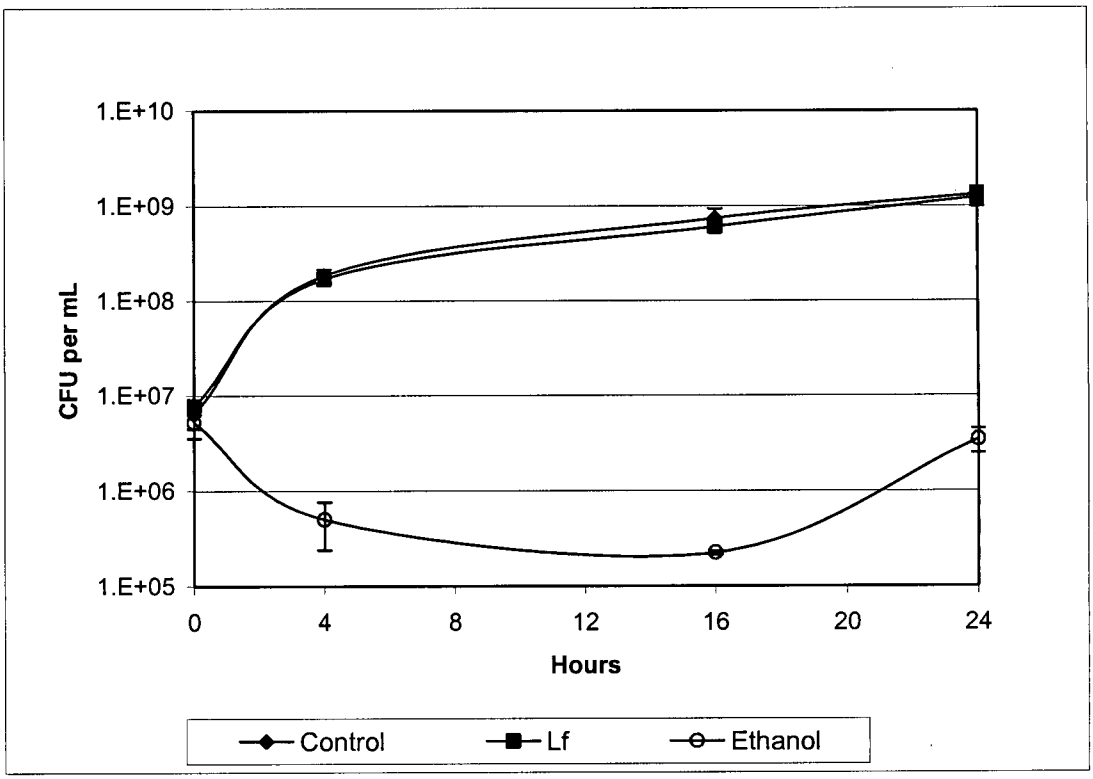


Figure 5.3. Effect of 10 mg mL^{-1}, lactoferrin (Lf) on *S.* Typhimurium survival in LB broth during 24 hours of exposure at 37°C. Counts obtained by plating on LB agar. The results are the mean value of three experiments. Bars represent standard deviation.

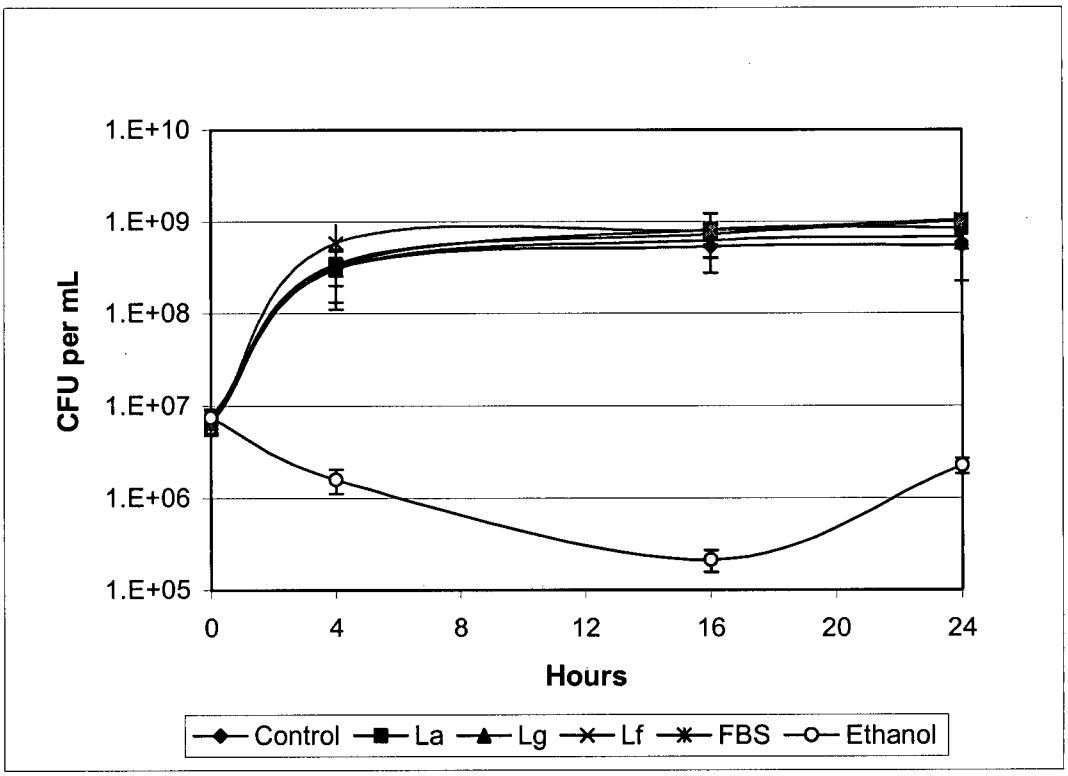


Figure 5.4. Effect of 15 mg mL^{-1} α-lactalbumin (La), β-lactoglobulin (Lg), lactoferrin (Lf) and fetal bovine Serum (FBS) on *S.* Typhimurium survival in LB broth during 24 hours of exposure at 37°C. Counts obtained by plating on LB agar. The results are the mean value of three experiments. Bars represent standard deviation.

bacteriostatic properties at the concentrations used (Araujo and Giugliano, 2001).

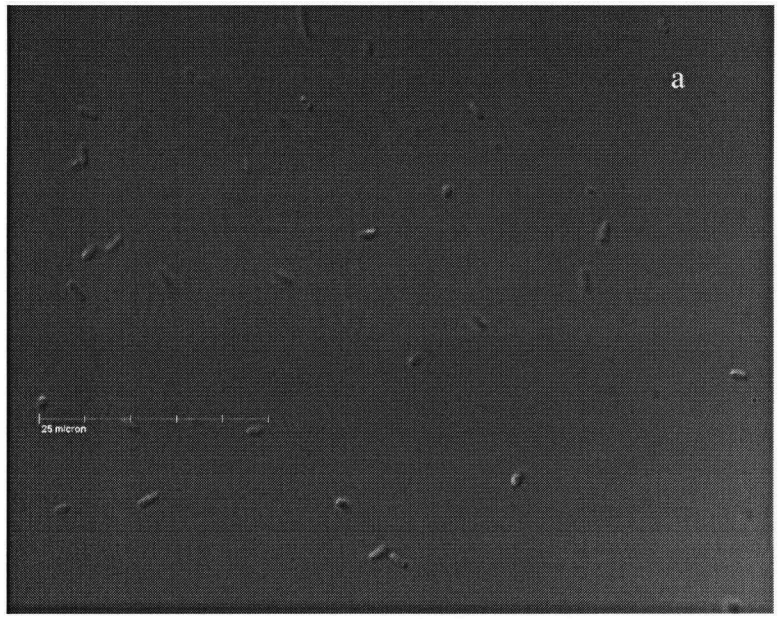
5.4.2 Aggregation of *S.* Typhimurium in the presence of whey proteins

Bacteria respond to their environmental conditions by transformation and morphology changes. Formation of bacterial cluster is one of the microbial behaviours in response to environmental changes. This phenomenon is defined as gathering of cells to form a fairly stable multicellular structure (Calleja, 1984). Researchers refer to this phenomenon using different terms such as microcolony formation (Drew, 1989), bacterial aggregation, agglutination, clumping, cohesion or agglomeration, to name a few (Calleja, 1984). In this thesis the term bacterial aggregation is used to point to this bacterial behaviour.

It has been stated that formation of bacterial aggregates prevents the bacteria from adhesion and subsequently invasion of tissue (Drew, 1989). Clumping of bacteria together reduces the number bacteria entering the mammalian cells simply because the aggregate will be too large and physically cannot be engulfed by the cell. To determine, if the observed lower bacterial counts in previous experiments was due to bacterial aggregation caused by whey proteins used in the invasion assay, *S.* Typhimurium was monitored using microscopy in the presence of lactoferrin, α-lactalbumin, β-lactoglobulin mixed variant, β-lactoglobulin A and β-lactoglobulin B at 10 and 15 mg mL^{-1}. To further extend our knowledge on the ability of those whey proteins in aggregating *S.* Typhimurium, monitoring continued for 24 hours and samples were taken at hours 0, 4, 16 and 24. Figure 5.5 shows the results of the

bacterial aggregation test. Figure 5.5a shows the negative control containing *S.* Typhimurium without protein treatment. Figure 5.5b is a typical representative aggregation experiment that was carried out with similar results for all tested whey proteins. All bacteria were individually spread throughout the microscope slide and no bacterial clumping was detected as a result of incubation with the selected whey proteins. Figure 5.5c represents *S.* Typhimurium treated with *S.* Typhimurium LPS-antibody and used as a positive control.

In a study with *Clostridium* species, Tomita, Hagiwara, Matsuyama and Kiyosawa (1998) reported the aggregation property of lactoferrin at 0.3 mg mL^{-1} as evidenced by absorbance measurement before adding the protein to microbial culture and after 5 minutes of incubation of lactoferrin with microbial suspension at room temperature. They also reported higher aggregate formation for bovine lactoferrin compared to human lactoferrin and attributed this difference to the structural divergence of these two lactoferrin types. Aggregating activity of lactoferrin on *Escherichia coli* O111 (Suzuki, Yamauchi, Kawase, Tomita, Kiyosawa and Okonogi, 1989), *Clostridium innocuum* and *Micrococcus luteus* has been reported. The authors also reported the hindrance of aggregation activity when lysine or arginine residues of lactoferrin were chemically modified. No aggregation activity was observed due to the presence of bovine β-lactoglobulin, α-lactalbumin and human α-lactalbumin (Tomita, Matsue, Matsuyama and Kiyosawa, 1994). Perraudin and Prieels (1982) also reported the aggregation of lysozyme-treated *Micrococcus luteus* by lactoferrin, which reached a plateau at 0.4 mg mL^{-1}.



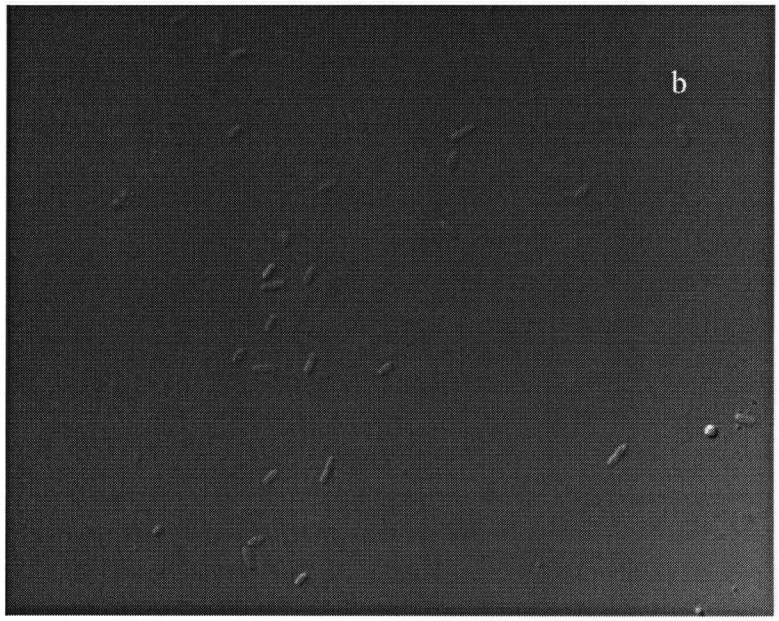


Figure 5.5. (See next page for legend)

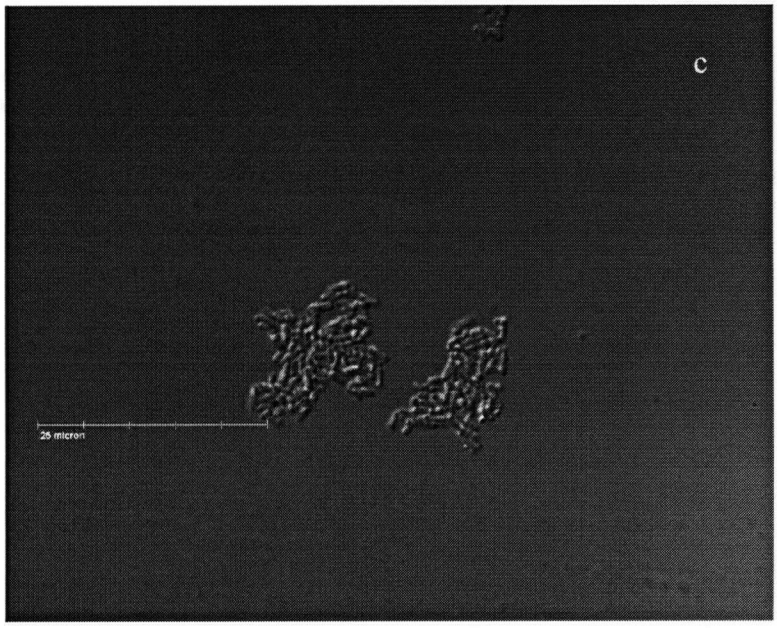


Figure 5.5. Micrographs of *S.* Typhimurium: a) untreated; b) typical representative *S.* Typhimurium treated with 10 and 15 mg mL^{-1} concentrations of bovine lactoferrin, α-lactalbumin, β-lactoglobulin (mix variant, A and B variants) during 24 hours of incubation at 37°C; c) treated with *Salmonella* LPS antibody

The aggregation was halted when 4 out of 39 lysine residues were modified, pointing to the possibility of electrostatic interaction between lactoferrin and bacteria.

These results suggest that since tested whey proteins were not capable of aggregating *S.* Typhimurium at 10 and 15 mg mL^{-1} during 24 hours of incubation, it can be concluded that the bacterial clumping was not responsible for the decreased level of invasion of HeLa cells by *S.* Typhimurium. As a consequence, the aggregation phenomenon was also eliminated from the possible invasion inhibition mechanisms.

5.4.3 Study the effect of pre-incubation of whey proteins with *S.* Typhimurium or HeLa cells on invasion inhibition

To further investigate the invasion inhibition process by whey proteins, the effect of pre-incubating whey proteins with either HeLa cells or *S.* Typhimurium prior to invasion assay was tested. *S.* Typhimurium suspension or HeLa cells were separately preincubated with 10 mg mL^{-1} of lactoferrin, α-lactalbumin and β-lactoglobulin followed by the invasion assay. As stated before, to make the comparisons easier and more conclusive, the bacteria, whey proteins and HeLa cell ratios were maintained constant in all procedures. Presence of binding activity between whey proteins and HeLa cell membrane was one of the speculated mechanisms for invasion inhibition. The binding could be either specific due to existence of particular binding sites or non-specific by binding to bacterial adhesion receptors on cell surface. The blocking of the bacterial adhesion receptors by whey

proteins may result in inhibiting the adhesion and consequently the invasion will be hindered. In the original method, HeLa cells were incubated with whey protein for 30 minutes before the invasion took place and once the bacterial culture was added, HeLa cells, bacterial culture and whey proteins were incubated for another 30 minutes. As shown in Table 5.1, despite an anti-invasive activity of 42.9% in the original method, anti-invasive activity of β-lactoglobulin significantly ($p<0.05$) decreased when a washing step was included in the procedure before adding the bacterial culture. Based on our results, it seemed that the binding activity between β-lactoglobulin and HeLa cells was not responsible for invasion inhibition and the protein was removed in the washing step before adding the *S.* Typhimurium suspension. Lactoferrin and α-lactalbumin showed anti-invasive activity of 45.9 and 37.6% respectively in the original method. Including the washing step before adding the bacterial suspension resulted in a much lower anti-invasive activity for both lactoferrin and α-lactalbumin. Since these whey proteins (lactoferrin and α-lactalbumin) to some extent were capable of conferring anti-invasive activity after washing of HeLa cells with PBS^{++}, it can be speculated that a portion of those whey proteins were retained after the washing step. This finding leads us to hypothesize that there is some binding activity between lactoferrin and α-lactalbumin and HeLa cells but not with β-lactoglobulin. Considering the fact that including the washing step resulted in lower anti-invasive activity, the assumption would be that binding was partially responsible for invasion inhibition by lactoferrin and α-lactalbumin and other mechanisms may also be involved.

Pre-incubation of lactoferrin, α-lactalbumin and β-lactoglobulin with *S.* Typhimurium prior to invasion, respectively, resulted in 65, 50 and 60% lower inhibition compared to the original procedure. Results suggest that pre-incubation of whey proteins with either HeLa cells or *S.* Typhimurium was ineffective or less effective in conferring anti-invasive activity compared to the original method in which HeLa cells were incubated with the test proteins for 30 minutes and *S.* Typhimurium culture was added and incubated with the protein-treated cells for another 30 minutes. Based on the results it is clear that simultaneous presence of whey proteins, HeLa cells and bacteria are necessary for the greatest inhibition of invasion to occur. This finding is in agreement with the work of Antonini and co workers (1997) who reported the inactivity of lactoferrin when it was pre-incubated with either bacteria or HT-29 and Caco-2 cells prior to invasion assay with *Listeria monocytogenes*. The same authors showed binding activity between lactoferrin with both *Listeria monocytogenes* and cell lines using particle agglutination assay. Their results were in agreement with the finding of other researchers who showed the binding of bovine lactoferrin to HeLa cells (Longhi, Conte, Bellamy, Seganti and Valenti 1994). In another study, researchers found that anti-adhesion activity of β-lactoglobulin was not due to binding to the bacteria. Pretreating of *Escherichia coli* with β-lactoglobulin did not affect the bacterial adhesion to human ileostomy glycoprotein (Ouwehand, Conway and Salminen 1995). They hypothesized that β-lactoglobulin binds to Sf aII receptor on human ileostomy glycoprotein and consequently prevents *Escherichia coli* from binding. Also a decrease (10-15%) in

adhesion of *S.* Typhimurium and *enteritidis* to human illeostomy glycoprotein in the presence of 50 $\mu g\ mL^{-1}$ β-lactoglobulin has been reported (Ouwehand, Salminen, Skurnik and Conway, 1997).

Another group of researchers reported the anti-invasive property of bovine lactoferrin against group A streptococci. They found that preincubating lactoferrin with bacteria or HeLa cells separately was not effective in inhibiting the streptococci invasion. Bovine lactoferrin ($1mg\ mL^{-1}$) significantly decreased the invasion of HeLa cells when the streptococci, HeLa cells and lactoferrin were present at the same time (Ajello, Greco, Giansanti, Massucci, Antonioni. and Valenti, 2002). As evidenced by our results, the mechanism of invasion inhibition is quite a complex process, the details of which have yet to be determined. Presence of specific lactoferrin-binding sites either on the cell envelope or porins of various bacteria has been reported (Erdei, Forsgren and Naidu, 1994; Schryvers and Morris, 1988; Naidu, Andersson, Miedzobrodzki, Forsgren and Watts, 1991; Naidu, Andersson and Forsgren, 1992). Confirming that binding activity is responsible for inhibiting *S.* Typhimurium from invading HeLa cells needs more specific investigations.

Table 5.1. The effect of pre-incubation of *S.* Typhimurium or HeLa cells with 10 mg mL^{-1} of selected whey proteins on *Salmonella* invasion of HeLa cells.

Anti-invasive activity after preincubation of whey proteins with			
	HeLa Cells		***S.* Typhimurium**
	Without washing*	**With washing**	
Control	$0.0^{a1} \pm 4.5$	$0.0^{a1} \pm 7.4$	$0.0^{a1} \pm 6.8$
β-lactoglobulin	$42.9^{b3} \pm 0.4$	$3.9^{a1} \pm 2.9$	$17.5^{b2} \pm 4.6$
α-lactalbumin	$37.6^{b2} \pm 0.9$	$22.3^{b1} \pm 4.4$	$19.3^{b1} \pm 0.9$
Lactoferrin	$45.9^{b2} \pm 5.5$	$23.1^{b1} \pm 3.7$	$16.2^{b1} \pm 2.0$

* Original invasion assay

Results represent mean values ± SD of 3 independent experiments with 3 replicates. P values calculated as compared to the control group and the level of significance was 0.05. Values with the same alphabetical superscript within each column are not significantly different. Values with the same numerical superscript within each row are not significantly different.

5.4.4 Inhibition mechanism theory

In this section the information provided by the results of experiments and collected literature were used to theorize mechanism(s) by which the selected whey proteins may confer the anti-invasive property.

It is known that *S.* Typhimurium invasion is regulated by specific bacteria host cell interaction. These interactions are accommodated by a bilateral signalling between bacteria and host cells, which results in cytoskeletal rearrangement on host cell and subsequently leads to forced pinocytosis of the bacteria. *Salmonella* signalling takes place through a secretion system. This system is called type III secretion system and is triggered by contact with host cells (Galán, 1996a; Johnston, Pegues, Huek, Lee and Miller, 1996).

By reviewing the invasion process, we may draw the conclusion that there are three main steps in each invasion process and interfering in each step may impede invasion. These three steps are bacteria-host cell contact, bacteria-host cell signalling and communication and finally macropinocytosis (Galán, 1996a). Preventing the macropinocytosis is a difficult if not impossible task, since once started it is not likely to stop. Preventing bacteria-host cell contact is possible either through eliminating the bacteria or by blocking the binding of bacteria to host cells. Our results showed that none of the tested whey proteins were able to eliminate the bacteria since the proteins were not bactericidal at the concentrations used. Also the tested whey proteins were not capable of effectively providing a physical barrier by promoting aggregation of *S.* Typhimurium. Blocking the binding sites on bacteria or bacteria receptors on cells

could be considered as one possible route for inhibition. Lactoferrin binding sites or receptors have been identified on cells and tissue surfaces (Naidu *et al.*, 1992; Alugupalli, Kalfas, Edwardsson, Forsgren, Arnold and Naidu 1994; Lönnerdal, 1994). Moreover because of a high pI (8.5-9.0) lactoferrin has been reported to interact with other macromolecules such as lipopolysaccharides, (Ellison and Giehl, 1991) lysozyme and β-lactoglobulin (Brock, 1997). A 57 kDa protein on cell envelope of 14 *Prevotella intermedia* strains, which bind lactoferrin in a dose-dependent manner, has been identified (Alugupalli *et al.*, 1994). The significance of these binding and interactions on biological function of lactoferrin has not been fully investigated. In the present research, including the washing step abolished the anti-invasive activity of β-lactoglobulin. Lactoferrin and α-lactalbumin retained some anti-invasive activity. This retention strongly suggests that lactoferrin and α-lactalbumin may bind to HeLa cells and resist the washing step. The question regarding how this binding affects *S.* Typhimurium invasion of HeLa cells remains to be answered. It can be speculated that lactoferrin and α-lactalbumin may bind to the *S.* Typhimurium receptors on HeLa cell surface and make the receptors unavailable for bacterial adherence and subsequent invasion. Another assumption would be that the binding only serves as a retaining factor and helps the proteins to remain in place and actual interaction takes place with the bacteria once the bacterial suspension is added to the HeLa cells.

Interfering with bacteria-host cell communication is another promising way of hindering the invasion process. During the signalling step, a number of proteins are secreted by *Salmonella* as a result of activation of the type III secretion system.

Although the molecular mechanism of exporting these proteins to host cell is still under investigation, it is probable that the *Salmonella* invasins and signalling molecules are injected into the host cells (Finaly and Flakow, 1997). Some of these proteins, with molecular weights ranging from 85 to 26 kDa, have been identified (Kaniga, Tucker, Trollinger, and Galán, 1995; Pegues, Hantman, Behlau, and Miller, 1995; Galán, 1996b). The function of these proteins are not fully understood, but it is clear that these proteins contribute to the signalling process either by helping the secretion or facilitating the function of signalling molecules and consequently result in host cell membrane rearrangement and bacteria engulfment (Galán, 1996a). Communication between host cell and *S.* Typhimurium results in formation of invosome, an appendage-like structure. This structure cannot be produced in growth media. Bacterial contact with live cells is essential in assembly of such a structure and chloramphenicol is unable to prevent the invosome formation (Galán, 1996b). Also several researchers reported that presence of type 1 pili is essential in invasion of *S.* Typhimurium (Horiuchi, Inagaki, Okamura, Nakaya and Yamamoto, 1992; Tavendale, Jardine, Old and Duguid, 1983).

Information in the literature on bacterial invasion procedure (Galán, 1996a; Galán 1996b; Finlay, 1994) leads to the speculation that any attempt in halting the communication between *S.* Typhimurium and host cell will result in inhibition of invasion. This can be achieved either by preventing the protein secretion or hindering the designated function of the secreted proteins.

As evidenced by the results it can be theorized that since washing rendered the β-lactoglobulin inactive in conferring anti-invasiveness it is possible that there is no strong physical blocking activity involved between HeLa cells and β-lactoglobulin and there should be another mechanism for inhibiting *S.* Typhimurium invasion. In the case of lactoferrin and α-lactalbumin, although the anti-invasive activity was significantly ($p<0.05$) lower after including the washing step, some anti-invasive activity was still observed. It can be concluded that invasion inhibition by lactoferrin and α-lactalbumin is different from β-lactoglobulin. Given the fact that lactoferrin has the potency of binding different molecules, it is possible that it may interfere with the protein(s) secreted for invasion signalling and derange their designated function. The validity of this theory should be further investigated.

5.5 Conclusion

Results showed that anti-invasive property of α-lactalbumin, β-lactoglobulin and lactoferrin was not due to bactericidal properties or bacterial aggregation. Changing the invasion condition and preincubating the whey proteins with either bacteria or HeLa cells and including a washing step resulted in decreased (lactoferrin and α-lactalbumin) or elimination (β-lactoglobulin) of anti-invasive activity. The results suggest that although these three whey proteins confer similar levels of anti-invasive activity at 10 mg mL^{-1}, the mechanism of inhibition seems to be different. Further studies will be necessary to establish the subtle mechanism by which these whey proteins inhibit *S.* Typhimurium invasion of HeLa cells.

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CHAPTER SIX

General discussion, general conclusion

and

recommendations for future studies

6.1 General discussion

S. Typhimurium is considered an important human and animal pathogen, which causes mild and acute gastroenteritis in humans. The infection can also lead to systemic form of disease and cause reactive arthritis and Reiter's syndrome. The pathogen is capable of cellular internalization by invading epithelial cells and consequently escaping host defence. The role of natural compounds in inhibiting microbial pathogenicity has been the subject of numerous studies amongst which a large proportion have been allocated to milk and milk components (van Hooijdonk, Kussendrager, and Steijns, 2000).

As mentioned earlier, consumption trend is towards utilization of foods which, besides their nutritive value and hunger satiety properties, can deliver a health promoting benefit. Considering this fact, the present research was designed to initiate studying the feasibility of incorporating whey proteins as functional food ingredients. It is reported here that bovine colostrum significantly diminished *S.* Typhimurium invasion in an *in vitro* HeLa cell model. There was evidence that the anti-bacterial activity of milk and colostrum was not limited to immunoglobulins. The anti-invasive activity of bovine immunoglobulins was previously investigated by Facon (1995). Ashkenazi and Mirelman (1987) demonstrated that the non-immunoglobulin fraction of human milk could inhibit adherence of enterotoxigenic *Escherichia coli* to intestinal cells.

To test the anti-invasive activity of the immunoglobulin G-free fraction of colostrum and also to narrow down the search for the active compound, skimmed

colostral whey was prepared and subjected to protein-G affinity column chromatography to exclude immunoglobulin G. The absence of immunoglobulin G in the unbound fraction was verified by sandwich ELISA and SDS-PAGE. The effect of the immunoglobulin G-free fraction on *S.* Typhimurium invasion of HeLa cell was tested using the gentamicin protection invasion assay. The results showed a considerable decrease in invasion level compared to the control, suggesting the presence of anti-invasive factor(s) in non-immunoglobulin G fraction of bovine colostral whey.

To elucidate the active compound(s), lactoferrin, α-lactalbumin, β-lactoglobulin and lactoperoxidase were tested for their anti-invasive activity. Lactoferrin, α-lactalbumin and β-lactoglobulin, at non-cytotoxic concentrations, successfully hindered *S.* Typhimurium invasion of HeLa cells in a concentration dependent manner. Anti invasive activity of lactoferrin has also been reported against group A streptococci (Ajello, Greco, Giansanti, Massucci, Antonioni, and Valenti, 2002) and *Listeria monocytogenes* (Antonini *et al.*, 1997). Lactoperoxidase displayed minimal anti-invasive activity in the present study.

Synergistic anti-bacterial effects was reported when combinations of milk proteins were used in treating animal infections with *Escherichia coli* (Van Leeuwen, Huisman, Kerkhof, and Kussendrager, 1998). Optimization techniques have been successfully used in formulation of food products with optimized characteristics (Dou, Toma, and Nakai, 1993). To study the feasibility of formulating a mixture with improved anti-invasive activity, Random Centroid Optimization (RCO) program was

used. Formulated mixtures of lactoferrin, α-lactalbumin and β-lactoglobulin were subjected to the invasion assay. Efficiency of the mixtures, as measured by percentage of anti-invasive activity per mg protein, was lower compared to individual proteins. The result suggested an antagonistic interaction between the whey proteins, which negatively affected anti-invasive activity. Binding activity between β-lactoglobulin with other molecules and also, molecular interaction of lactoferrin and β-lactoglobulin has already been reported (Lampreave, Pineiro, Brock, Castillo, Sanchez, and Calvo, 1990). There is a possibility that these molecular interactions are responsible for the observed antagonistic effect. The details of the mechanism by which these proteins interact and the effect of these interactions on anti-invasive activity should be further investigated.

To delve more into the mechanism by which the selected whey proteins inhibit the *S.* Typhimurium invasion of HeLa cells, the proteins were tested for bactericidal and bacterial aggregation properties. Proteins were neither bacteriostatic nor capable of aggregating the bacteria at the concentrations and durations used. Preincubation of bacterial culture prior to invasion did not improve anti-invasive activity of any of the whey proteins used. Including a washing step prior to invasion eliminated the anti-invasive activity of β-lactoglobulin and reduced the activity of lactoferrin and α-lactalbumin. The results may reflect the presence of different mechanisms by which whey proteins inhibit HeLa cell invasion by *S.* Typhimurium. The question regarding how the difference in invasion percentage relates to structural differences of whey proteins remains to be answered.

Although the mechanism by which the selected whey proteins inhibit invasion is not clear, based on our results, a number of theories have been postulated. The hypothesis that the lower recovered CFUs of *S.* Typhimurium in protein treated samples after invasion assay is due to bacteriostatic, bactericidal activity or aggregation prior to invasion was excluded. Results of modifying the invasion assay to include preincubation of whey proteins with either bacteria culture or HeLa cells suggest that the simultaneous presence of HeLa cells, bacterial culture and whey proteins are required. Several research studies with lactoferrin show that this protein is able to bind various host cells (Antonini, *et al.*, 1997; Davidson, and Lönnerdal, 1988) and bacteria (Antonini *et al.*, 1997; Visca, Dalmastri, Verzili, Antonini, Chiancone, and Valenti, 1990). Lactoferrin receptors have been identified on the surface of various bacteria such as *Streptococcus pneumoniae* (Häkansson, Roche, Mirza, McDaneil, Brooks-Walter and Briles, 2001) and *Neisseria gonorrhoeae* (Lee and Schryvers, 1988). Despite the evidence for existence of binding interaction between whey proteins, especially lactoferrin and host cells, our results show that this binding is only partially responsible for the observed anti-invasive activity of lactoferrin and α-lactalbumin. Based on our results, it is clear that whey proteins interfere with the bacteria-host cell communication and halt the invasion. The inhibition can be achieved through different routes. It is possible that whey proteins act as bacterial receptor analogs and prevent the binding of bacteria to the host cell receptors by blocking the binding sites. Also it can be speculated that whey proteins bind to host cell receptors and make them unavailable for bacterial binding.

Interaction of whey proteins with the function of type III secretion system can also interfere with the invasion process. This secretion system is responsible for signalling and transferring invasion messages from bacteria to host cells and consequently initiation of invasion (Finlay and Falkow, 1997). Researchers reported that glycoconjugates may interfere with the expression of the proteins required for adherance of enteropathogenic *Escherichia coli* (EPEC) and prevent adherence (Vanmaele, Heerze, and Armstrong, 1999). To determine if any of these mechanisms or their combination are responsible for observed inhibition requires more advanced investigation.

6.2 General conclusion

In the present study, we discovered for the first time a new biological function for bovine α-lactalbumin and β-lactoglobulin. The results show that whey proteins namely lactoferrin, α-lactalbumin and β-lactoglobulin possess significant anti-invasive activity against *S.* Typhimurium at non-cytotoxic and non-bactericidal concentrations. The inhibitory effect of whey proteins on *S.* Typhimurium invasion of HeLa cells is likely to be a complex process involving a variety of factors.

Since invasion is an essential step for pathogenesis of *Salmonella*, the results of the present study suggest that whey proteins have the potential of preventing disease caused by *Salmonella*. Also it has been shown that this inhibitory effect is not due to impeding *S.* Typhimurium growth or aggregation of the bacteria. Moreover, the anti-invasive activity of the mixture of the proteins was not additive and the results of

optimization experiments suggested a complex antagonistic interaction, which affected the anti-invasive activity of the three whey proteins tested in this study.

In conclusion data were obtained for the first time, showing that whey proteins are able to inhibit the *S.* Typhimurium invasion of HeLa cells. At this point, the goal of this research, which was to investigate the anti-invasive property of whey proteins, was met. The results may serve as a preliminary study for further inclusion of these compounds in health-promoting functional foods. The results strongly suggest an important biological function for whey proteins and we are one step closer to finding evidence that whey proteins have the potential of being considered as functional food ingredients for therapeutic or preventative purposes. The result of the present study showed a difference between anti-invasive activity imparted by β-lactoglobulin A and B. Also RCO program was used for the first time to investigate the anti-invasive activity of the mixtures of the proteins.

6.3 Recommendations for future studies

Although I strongly believe that whey proteins have the potential of being used as bacteria fighting food ingredients, there are numerous factors, which should be taken into consideration, before any claim should be substantiated. Processing may greatly affect any food ingredient including the ingredients, which aim to deliver special health benefits. Without the comprehensive knowledge of what exactly happens to these valuable components they may be lost during processing. I suggest examining the effect of food processing, especially thermal processing on anti-

invasive activity of whey proteins. The results may provide useful information on process directions. It has been reported that the anti-adhesion activity of β-lactoglobulin was diminished by boiling in presence of 2% mercapto-ethanol (Ouwehand, Salminen, Skurnik, and Conway, 1997). Research should be aimed at finding interaction between processing conditions and presence of other ingredients in the mixture on the anti-invasive activity of whey proteins.

Moreover, whey proteins should retain their molecular structure until they reach the intestinal tract in order to confer their anti-bacterial effect. Hence it is important to examine the stability of the whey proteins in gastric environment in order to choose an appropriate delivery system and control the release of the bioactive compound in the desired part of gastrointestinal tract. The stability of any substance is highly correlated to the matrix in which it is delivered. In a study using murine model, researchers found that lactoferrin in milk was more resistant, than the pure protein, to gastric juice (Dial, Hall, Serna, Romero, Fox, and Lichtenberger, 1998). It is recommended to test the effect of gastric juice and bile salts on the anti-invasive activity of whey proteins both in pure form and in a selected food formulation under a simulated gastric environment.

Structural conformations of proteins are intrinsic to their function and biological activity. Researchers found that folding state of α-lactalbumin causes apoptosis in tumour cells, while the native form was ineffective. This conformation can also take place in the acid environment of the stomach (Svensson, Häkansson, Mosseberg, Linse, and Svanborg, 2000). Thus it is important to study the structural

conformation of whey proteins and to test their anti-invasive activity under different conditions mimicking the human digestive tract environment or in a food matrix.

In order to have more practicality, it is suggested that the anti-invasive property of the food grade preparations of the individual whey proteins versus the analytical grade be evaluated. To elucidate the role of iron in anti-invasive activity and also to determine the structural effect, it is recommended to examine iron-saturated and iron-free lactoferrin and also ovotransferrin, human plasma transferrin and lactoferrin from other species for their anti-invasive activity.

Researchers found that using lactoferrin enhanced the efficacy of antibiotics, in sublethal concentrations, in inhibiting streptoccal invasion of HeLa cells (Ajello *et al.*, 2002). This issue should be further investigated to determine the synergistic effect of whey proteins in antibiotic therapies.

There are a few reports on anti-invasive activity of lactoferrin against different bacterial strains such as *Listeria*, enteroenvasive *Escherichia coli* and streptococci. It would be also interesting to investigate the anti-invasive activity of lactoferrin on a broader range of invasive bacteria and also to the test anti-invasive activity of α-lactalbumin and β-lactoglobulin on other invasive bacteria.

While lactoferrin, α-lactalbumin and β-lactoglobulin demonstrated the ability to inhibit *S.* Typhimurium invasion *in vitro*, care must be taken in extrapolation of this observation to *in vivo* conditions. Although regulation of natural products has recently become an issue of concern and even though some natural anti-bacterial agents such as lactoferrin are already being incorporated in food in some countries, there should

be enough well controlled and well defined *in vivo* studies to support the effectiveness and safety of these components before they find their way into the consumer market.

6.4 References

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