

ENHANCEMENT OF INNATE IMMUNE DEFENSE BY
SUPPLEMENTATION WITH PRESSURIZED WHEY
PROTEIN

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March 2012

A thesis submitted to the Faculty of Graduate studies and Research in partial fulfillment
of the requirements for the degree of
DOCTOR OF PHILOSOPHY

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This thesis is dedicated to the memory of my father, Abdulla N. Kishta,
my mother F. Kishta, my wife Linda, my daughters Noor, Nuha,
Leena and Sara, and my uncle Galal

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ABSTRACT

Chronic pulmonary infection with *Pseudomonas aeruginosa* (*P. aeruginosa*) in patients with cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) is associated with persistent inflammation, exuberant oxidative stress, increased mortality and morbidity. Studies showed that whey protein (a byproduct of the cheese-making process) or its components can enhance innate immune functions of neutrophils *ex vivo* and decrease bacterial metabolic activity *in vitro*. Whey-derived peptides enhanced innate defense functions of neutrophils *ex vivo* and protected mice against infection. Treatment of whey protein with hyperbaric pressure increases its digestibility, alters the spectrum of peptides available for systemic absorption, and enhances its antioxidant potential. Hence the overall objective of this thesis was to test the hypothesis that supplementation with pressurized whey protein can enhance the host's ability to clear microbial infection more efficiently compared to native whey. To this end we used a murine model of sustained pulmonary infection with *P. aeruginosa*. Our time course study demonstrated that both lung infection and inflammation (indexed by inflammatory cell count in lung lavage fluid, body weight loss and myeloperoxidase activity in lung tissue) peak or start to diminish at Day 3, then wane by Day 7 while airway lumen protein oxidation persists. Two groups of female C57BL/6 mice were then randomized to receive either native or pressurized whey-based diets for four weeks, after which they were infected with 8×10^5 CFUs/mouse of *P. aeruginosa*. Bacterial burden at Day 3 was 13-fold less in the pressurized whey group ($p < 0.5$) without associated changes in the inflammatory response. Further approaches were then undertaken to explore the mechanisms of

these findings. We found that supplementation with pressurized whey increased airway lumen neutrophil bactericidal activity and superoxide anion production, reflecting a higher innate defense capacity. Airway protein oxidation was also reduced in the pressurized whey group. Airway pro-inflammatory cytokines also suggested reduced local responses in the pressurized whey group, with a 10-fold less granulocyte-macrophage colony stimulating factor and trends towards lower interleukin-1 β and macrophage inflammatory protein-2. In conclusion, supplementation with pressurized whey protein can enhance innate immune defenses against microbes to a greater extent than native whey and represents a promising nutritional approach towards enhanced immune protection from infection.

RÉSUMÉ

Les infections chroniques pulmonaires causées par *Pseudomonas aeruginosa* (*P. aeruginosa*) chez les patients atteints de fibrose kystique (FK) ou de maladies pulmonaires obstructives chroniques (MPOC) sont associées à une inflammation persistante, un stress oxydatif exubérant, ainsi qu'une mortalité et une morbidité accrues. Des études ont montré que les protéines du lactosérum (un sous-produit du processus de fabrication du fromage) ou ses composantes peuvent améliorer les fonctions immunitaires innées des neutrophiles *ex vivo* et peuvent diminuer l'activité métabolique des bactéries *in vitro*. Les peptides dérivés du lactosérum améliorent les mécanismes de défenses innées des neutrophiles *ex vivo* et protègent les souris contre l'infection. Le traitement des protéines du lactosérum avec une pression hyperbare augmente leur digestibilité, modifie le spectre de peptides disponibles pour l'absorption systémique et améliore son potentiel antioxydant. Donc l'objectif global de cette thèse était de tester l'hypothèse selon laquelle la supplémentation du lactosérum en protéines par pressurisation peut améliorer la capacité de l'hôte à éliminer l'infection microbienne par rapport au lactosérum natif. À cette fin, nous avons utilisé un modèle murin d'infection pulmonaire soutenue, causée par *P. aeruginosa*. Notre étude de décours temporel a démontré que l'infection pulmonaire et l'inflammation (indexés par le nombre de cellules inflammatoires dans le liquide de lavage pulmonaire, la

perte de poids corporel et l'activité myéloperoxydase dans le tissu pulmonaire) culmine et commence à diminuer au jour 3, puis décroît jusqu'au jour 7 tandis que l'oxydation des protéines de la lumière des voies respiratoires persiste. Deux groupes de souris C57BL / 6 femelles ont ensuite été randomisés pour recevoir soit une diète à base de lactosérum natif ou à base de lactosérum supplémenté par pressurisation pendant quatre semaines, après quoi ils ont été infectés avec 8×10^5 UFC (unité formant une colonie) / souris de *P. aeruginosa*. La charge bactérienne au Jour 3 était 13 fois moins importante dans le groupe ayant reçu le lactosérum pressurisé et ce sans changement associé au niveau la réponse inflammatoire. D'autres approches ont alors été considérées afin d'étudier les mécanismes pouvant expliquer ces résultats. Nous avons constaté que le groupe ayant reçu une diète à base de lactosérum pressurisé, présentait une augmentation de l'activité bactéricide des neutrophiles présents dans la lumière des voies respiratoires, accompagné d'une production accrue d'anion superoxyde, traduisant une capacité de défense innée amplifiée. L'état d'oxydation des protéines des voies respiratoires a également été réduit dans le groupe ayant reçu le lactosérum pressurisé. Les cytokines pro-inflammatoires présentent dans les voies respiratoires suggèrent une réponse inflammatoire locale réduite dans le groupe ayant reçu le lactosérum pressurisé. En effet le facteur stimulant les colonies de granulocytes-macrophages est réduit par un facteur 10 et une tendance à la baisse est aussi observée pour l'interleukine-1 β et la protéine inflammatoire des macrophages-2. En conclusion, la supplémentation avec de protéines de

lactosérum pressurisé peut augmenter les défenses immunitaires innées contre les microbes dans une plus grande mesure que le lactosérum natif et représente une approche nutritionnelle prometteuse afin d'accroître la protection immunitaire contre l'infection.

ADVANCE OF SCHOLARLY KNOWLEDGE

1. Claims of novel findings:

The thesis has proved that supplementation with pressurized whey protein enhances the innate immune defense of the host to control prolonged lung infection more efficiently than native whey protein. The results from this thesis work have demonstrated novel innate immune-modulating effects of pressurized whey protein by showing that:

1. Protein oxidation in the bronchoalveolar lavage fluids of mice with prolonged pulmonary infection with *P. aeruginosa* is increased at Days 1, 3, and 7 post-infection as compared to non-infected controls; this was associated, in parallel, with a tendency towards decreased levels of the antioxidant glutathione. These findings reflect increased airway oxidative stress in this animal model of infection, mimicking the oxidative challenge in infected CF patients. These results suggest that the anti-oxidant capacities of pressurized whey may be beneficial in such lung infections.
2. Supplementation of mice with pressurized whey-based diets results in lower levels of lung bacterial burden 3 days following infection with *P. aeruginosa* (using a prolonged infection model), as compared to native whey-fed controls.
3. Mice pre-fed with pressurized whey protein exhibit decreased airway protein oxidation compared with native whey-fed controls.

4. Neutrophils from pressurized whey protein pre-fed mice have higher bactericidal activity and higher superoxide anion production, suggesting a stronger respiratory burst.
5. The airways of mice pre-fed with pressurized whey protein exhibit lower concentrations of the inflammatory cytokine granulocyte-macrophage colony-stimulating factor, and trends towards decreased concentrations of the inflammatory cytokines, macrophage inflammatory protein 1 and interleukin-1 β .
6. Presenting preliminary evidence that supplementation with pressurized whey protein enhances neutrophil count in blood in response to bacterial infection, which could be hypothesized as a strengthened line of innate immune defense against bacteria by pressurized whey protein.

2. Research manuscripts submitted to refereed journals or in preparation for submission:

1. O.A. Kishta, N. Dauletbaev, and L.C.Lands. Time Course of Inflammatory Response, Infection and Oxidant Damage in a Murine Model of Chronic Lung Infection with *Pseudomonas aeruginosa* (in preparation for submission to : Immunobiology)
2. O.A. Kishta, M. Iskandar, N. Dauletbaev, S. Kubow and L.C. Lands. Supplementation with Pressurized Whey Protein Limits *Pseudomonas aeruginosa* Lung Infection in Mice (in preparation for submission).

3. Abstracts

1. O. Kishta, M. Skandar, L.C. Lands. Effect of pressurized whey protein in a murine model of *Pseudomonas aruginosa* lung infection. Am. J. Resp. Crit. Med. 181:A1791. (ATS: American thoracic Society, May 2009).
2. O. Kishta, N. Dauletbaev, L.C. Lands. Time course of lung infection with *Pseudomonas aruginosa* : contribution of Oxidative stress. Am. J. Resp. Crit. Care Med. 179: A2845 (ATS, May 2009)
3. O.Kishta, N. Dauletbaev, L.C. Lands. Protein oxidation in lung infection with *Pseudomonas aruginosa*. Am J. Resp. Crit. Care Med. 181:A1791 (ATS, 2010)

PREFACE

In accordance with the “Guidelines for thesis preparation” of the faculty of Graduate Studies and Research at McGill University, and as advised by my supervisor, Dr. L.C. Lands and my committee members (Dr. J. Martin, Dr. C. McCusker, and Dr. J. Rak), I opted to present my research thesis in a traditional narrative style, with the addition of data not included in the two manuscripts.

The whole thesis includes seven chapters. Chapter 1 includes introduction, rationale, hypothesis and objectives of the research project; Chapter 2 includes a detailed review of literature. In Chapter 3, I present a background about the animal model used in my studies followed by a description of the methods in this model of pulmonary infection. Chapter 4 includes a time course study of the infection. Chapter 5 includes a preliminary study and the principal study of the effect of pressurized whey in enhancing innate immune defences and the mechanisms behind the anti-bacterial effect of pressurized whey protein. In Chapter 6, I present preliminary evidence for a new avenue in the mechanistic aspects of enhanced innate immunity by supplementation with pressurized whey protein. Chapter 7 includes an overall summary, limitations, and future directions.

CONTRIBUTION OF CO-AUTHORS TO MANUSCRIPTS

This thesis involved the collaboration of the primary supervisor, Dr. Lands, Pediatric Respiratory Medicine, The Montreal Children's Hospital, Montreal, Quebec, and Dr. Kubow, School of Dietetics and Human Nutrition, Macdonald Campus of McGill University, who together, originated the project, and provided experience in whey-based diets.

The candidate, author of this thesis, designed and performed the experiments in accordance with the objectives of the project and following the conclusions of the preliminary study. The candidate also derived hypotheses for the mechanistic aspects of the enhanced innate immune defense by pressurized whey protein, suggested most parameters, and chose methods for most of the measurements. He was also responsible for diet preparation, animal feeding, inoculum preparation, infection and animal harvest, most of the measurements, analyses and interpretation of data. He drafted and wrote the manuscripts and the literature review as well.

Dr. Lands supervised and critically reviewed the suggested parameters and data analyses, held sessions to follow up the progress, suggested helpful parameters, analyses and solutions to the candidate, whenever needed. He also helped in data interpretation, gave feedback and advice to the candidate throughout the whole of the thesis work, critically reviewed the manuscripts and literature review, and provided editorial comment.

Dr. Kubow helped in the pressurized whey study where he provided the design of the diet, and provided the candidate with views in the analyses of the primary_outcomes. He also provided editorial comment for the manuscript entitled

“Supplementation with Pressurized Whey Protein Limits *Pseudomonas aeruginosa* Lung Infection in Mice” and the literature review

Dr. Michele Iskandar counted bronchoalveolar lavage cells from mice sacrificed on Day 3 of the pressurized whey feeding study, prepared slides for differential cell counts, and de-proteinized bronchoalveolar lavage supernatants in preparation for glutathione measurements. Dr. Nurlan Dauletbaev adapted the method for total glutathione measurement in bronchoalveolar lavage supernatants and measured this parameter in samples from the time course and pressurized whey feeding studies.

ACKNOWLEDGEMENT

I would like to extend my deepest gratitude to my advisor Dr. Larry Lands for the chance he gave me in his laboratory, his patience, high scientific standards, advice, critical review, unconditional support and confidence that made this accomplishment come to light. Throughout my interactions with Dr. Lands, I learnt from him an approach to critical review and statistical analysis that significantly contributed to my skills and views as a scientist.

Sincere thanks are also extended to my thesis committee members, Dr. J. Martin, Dr. C. McCusker, and Dr. J. Rak for their instructions, critical review, and guidance throughout the different stages of my PhD project.

I would like to thank Dr. Hugh Bennett, Director of Division of Experimental Medicine for his effort in founding and organizing this division as well as his support of the graduate students.

Thanks are extended to Dr. Stan Kubow for his advice and views he offered me and for editing the literature review and pressurized whey feeding manuscript. I would like to thank my colleagues and co-workers at Dr. Lands' laboratory, MCH, Dr. Nurlan Dauletbaev for his help, guidance, cooperation, and scientific interactions throughout the project and for editing the final versions of the manuscripts; and Dr. Michele Iskandar for supervising me in diet preparation, her assistance in the preliminary study, scientific interactions, and editing the final version of this thesis.

I would like to thank Dr. Qutayba Hamid, director of the Meakins-Christie laboratories, and Normand Lavoie, coordinator of the same facility, for the generous access to the facility in order to perform the bacterial protocols and experiments.

I would like also to thank Karim Shalaby, PhD candidate, Dr. Martin's Laboratory, Julie Berube, research assistant, Dr. Rousseau laboratory, Johanne Bourdon, chief technician, Dr. Petrof's laboratory, and Dr. Jichuan Shan, research associate, Dr. Fixman's laboratory, all at Meakins-Christie laboratories, for their support and cooperation, particularly in facilitating access to the equipments in their laboratories.

I would like to extend my sincere thanks and deep gratitude to Doris Greinke, ex-supervisor at the McIntyre animal facility, McGill University; her distinctive support during the establishment of the infection method and throughout the performance of the experiments was critically helpful. Thanks are also extended to Anna Jimenez, chief animal health technician at the same facility, for her kind support, technical help and advice. I would like also to thank Gill Fruchter, undergraduate trainee, for his technical and logistic assistance.

I would like to thank my family members for their support and courage throughout the PhD endeavor, in particular my mother, father, my wife, Linda, my daughters Noor, Nuha, Leena and Sara, and my uncle Galal. Without their love and support, none of this would be achieved.

The one-year studentship and the associated course work from the Quebec Respiratory Health Training Program (QRHTP) were highly appreciated. I also highly appreciated a 3-year scholarship generously offered by Bourse de formation Doctorate, Fonds de la recherche en Sante du Québec (FRSQ). A travel award from Division of Experimental Medicine is also recognized and appreciated. This work was supported by a grant from the Toronto Sickkids Foundation.

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LIST OF ABBREVIATIONS

α - LA: Alpha-Lactalbumin

APPs: Acute phase proteins

ATPase-dependent: Adenosine Triphosphatase-dependent

BPI: Bactericidal/permeability increasing protein

BALF: Bronchoalveolar lavage fluid

β - LG: Beta-Lactoglobulin

CF: Cystic Fibrosis

CFTR: Cystic Fibrosis Transmembrane Conductance Regulator

CF fMLP: formyl Methionyl-Leucyl-Phenyl alanine

ENaC: Epithelial sodium channel

GMP: Glycomacropeptide

GLF: Glycyl-Leucyl-Phenylalanine

GM-CSF: Granulocyte Macrophage-Colony Stimulating Factor

GSH: glutathione

HIV: human immunodeficiency virus

H₂O₂: Hydrogen peroxide

HOCl: Hypochloric acid

HNE: 4-Hydroxy-2-nonenal

IL-1 β : Interleukin-1 β

IL-6: Interleukin-6

IL-5: Interleukin-5

IL-13: Interleukin-13

INF- γ : Interferon- γ

IL-4: Interleukin-4

KC: Keratinocyte Chemoattractant

LF: Lactoferrin

LPS: Lipopolysaccharide

MIP-2: Macrophage inflammatory protein-2

NADPH: Nicotine adenine dinucleotide phosphate

nWPI: native whey protein isolate

O₂ - : superoxide radical

PMA: Phorbol Myestrate Acetate

pWPI: Pressurized whey protein isolate

P. aeruginosa: *Pseudomonas aeruginosa*

SOD: Superoxide Dismutase

Th1: T helper 1

Th2: T helper 2

TNF- α : Tumor necrosis factor- α

TLR-4: Toll-like receptor 4

TLR-5: Toll-like receptor 5

TSB: tryptic soy broth

TTSS: Type III secretion system

Stat 6: Signal transducer and activator of transcription 6

WPI: Whey Protein Isolate

WT: wild type

WPC: Whey protein concentrate

CHAPTER 1

INTRODUCTION

1.1. Rationale and hypothesis:

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic pathogen that infects immune-compromised patients such as transplant recipients, neutropenic patients and patients with human immunodeficiency virus (HIV). It is also the most common pathogen responsible for chronic respiratory infections in Cystic Fibrosis (CF) patients (1-4), in addition to patients with other obstructive pulmonary diseases such as Chronic Obstructive Pulmonary Disease (COPD) (4-6) and bronchiectasis (4).

CF patients are more vulnerable to infection with *P. aeruginosa* due to their viscous, thick and dehydrated layers of mucus secretions in the airways, impairing mucociliary clearance of bacteria. Pulmonary infection with *P. aeruginosa* causes exuberant oxidative stress via a variety of mechanisms (7-10). This oxidative stress can impair neutrophil killing ability of the microbes. The physiological oxidant, hydrogen peroxide, inhibits phagocytosis and bactericidal activity of human neutrophils *in vitro* (11). *P. aeruginosa* can also directly inhibit the neutrophils' bacterial killing ability through the secretion of extracellular alkaline protease and elastases, since both of these bacterial proteases have been shown to inhibit the oxygen-dependent antimicrobial system in human neutrophils *in vitro* (12). In addition, *P. aeruginosa* infection induces inflammation via its virulence factors while resisting or circumventing host defense mechanisms. This resistance is even more challenging in the case of chronic infection with the mucoid strain of this pathogen,

which grows in a biofilm, as exemplified by the chronic infection that takes place in CF patients with *P. aeruginosa*.

Whey protein is a dairy by-product of the cheese-making process. Whey is characterized by a rich content of essential amino acids and is known for its health benefits (13). Whey protein extracts were found to enhance superoxide anion production by both sheep and human neutrophils (14, 15) as well as chemotaxis and phagocytosis of bioparticles by human neutrophils *in vitro* (14). Peptides from whey proteins protected mice from *Klebsiella pneumonia* infection (16). Hydrolysates of pepsin- or trypsin-digested whey protein components (β -lactoglobulin, β -LG) or (α -lactalbumin, α -LA) decreased the metabolic activity of *Escherichia coli* to a greater extent than the native, undigested components (17). Likewise, pepsin-digested glycomacropeptide (GMP), a component of whey protein, enhanced the proliferation and phagocytic activity of a macrophage-like cell line to a greater degree than undigested GMP (18). These studies indicate that hydrolysis products of whey components have enhanced biological effects compared to the native proteins.

Hyperbaric pressure increases the digestibility of whey proteins and alters the peptide profile of the resulting digestates (19), which may have immune-modulating effects and enhance host defense mechanisms. Hyperbaric pressure also enhances the antioxidant potential of whey protein (18). Therefore we hypothesize that pressurization of whey proteins will enhance their anti-bacterial effects.

Rationale:

Pulmonary infections with *P. aeruginosa* result in lung damage due to persistent inflammation and oxidative stress. Exposure of whey protein to high hydrostatic pressure leads to enhanced digestibility and alters the peptide profile of the resulting hydrolysates, improving their ability to enhance innate immune function and protect against oxidative damage.

Hypothesis:

We hypothesize that supplementation with pressurized whey protein enhances the innate immune defense against bacterial infection to a greater extent than native, unpressurized whey protein.

1.2. Overall objective and specific aims:**Overall objective:**

The overall objective of this research project is to test whether supplementation with pressurized whey protein enhances innate immune defenses in a murine model of chronic lung infection with *P. aeruginosa*, more efficiently than its native unpressurized counterpart.

Specific aims:

- 1) To identify the time course of the inflammatory response, infection and protein oxidation in the lung lavage of *P. aeruginosa*-infected C57BL/6 female mice and control mice exposed to sterile beads.

- 2) To study the inflammatory response and lung bacterial burden in mice pre-fed with native whey or pressurized whey protein followed by pulmonary infection with *P. aeruginosa*.
- 3) To study the potential mechanisms behind the expected attenuated inflammatory response and/or enhanced innate defense in the pressurized whey-fed mice.

CHAPTER 2

REVIEW OF LITERATUTRE

2.1. *Pseudomonas aeruginosa* (*P. aeruginosa*):

P. aeruginosa is a ubiquitous gram negative aerobic bacterium, measuring 0.5-0.8 μm by 1.5-3 μm and belongs to the bacterial family *Pseudomonadaceae*. Although it is an aerobic bacterium, it can survive and grow under anaerobic conditions if there are sufficient amounts of nitrate (NO_3^-) as an electron donor. Its metabolism is respiratory and not fermentative. *P. aeruginosa* exists in three forms in nature: planktonic bacteria, living as unicellular organisms swimming via their flagella, attached to any surface, or in biofilms as in the case of chronic infection.

It is an opportunistic pathogen, which exploits any weakness in the immune system to initiate infection. It is the most common pathogen responsible for respiratory infections in ventilated or immune-compromised patients such as transplant recipients, neutropenic patients and patients with HIV (20). In addition, Cystic Fibrosis (CF) patients are vulnerable to infection with *P. aeruginosa* due to the accumulation of viscous and dehydrated mucus secretions in their airways, leading to impaired mucociliary clearance of bacteria. There is evidence that *P. aeruginosa* causes exuberant oxidative stress in the airways. Suntres and colleagues (10) found that infecting rats intra-tracheally with *P. aeruginosa* dramatically increased lipid peroxidation, and decreased both the antioxidant thiol, glutathione, and antioxidant enzyme activities (superoxide dismutase, catalase, glutathione peroxidase and disulfide glutathione reductase) in lung homogenates at 3 days post-infection.

2.1.1. Virulence factors of *P. aeruginosa*:

P. aeruginosa expresses virulence factors on its cell membrane for adherence with the host epithelium, in addition to the extracellular products, which are produced by the bacteria after infection or colonization and cause extensive tissue damage, dissemination and inflammation. The most studied cell membrane and extracellular virulence factors and their biological effects, including those associated with chronic lung infection with *P. aeruginosa*, are:

2.1.1.1. Bacterial surface factors:

Bacterial surface factors such as pilus and lipopolysachharide (LPS) on the outer surface of bacteria, which binds Toll-like receptor-4 (TLR-4) and polar flagella, which binds Toll-like receptor-5 (TLR-5); both of these receptors are expressed on epithelial cells and macrophages, where they bind with their ligand on the bacterial surface resulting in early innate immune response via activation of NF- κ B in these cells (46).

2.1.1.2. Pyocyanin:

This is a blue colored redox phenazine pigment (1-hydroxy-methylphenazinium hydroxide), a metabolite of *P. aeruginosa* which gives the bacterial colonies a blue-green color (21). Pyocyanin causes damage to lung tissue through oxidative stress, amongst various mechanisms. It has been reported to inhibit catalase activity in human lung epithelial cells (8), which explains, at least in part, the reported oxidative stress in lung tissues of rats infected with *P. aeruginosa* (10). In this context, incubation of endothelial cells with pyocyanin increased extracellular hydrogen peroxide, decreased total intracellular levels of both glutathione and soluble

thiols (protein-SH and non-protein) (7), Given that endothelial cells are one of the compartments of lung parenchyma, this supports that lung parenchymal cells contribute to the oxidative stress reported in the lung homogenates of *P. aeruginosa*-infected animals, reported above (10). Pyocyanin also decreases or compromises the host's defense against microbes, through various mechanisms. For example, pyocyanin inhibits macrophage production of nitric oxide, a recognized component of host defense against several pathogens (22). It was also reported to induce neutrophil apoptosis in a concentration and time dependent manner, suggesting the role of this inhibition in the persistence of infection (23). Pyocyanin has been recently found to block the killing of *P. aeruginosa* by the DUOX/thiocyanate/lactoperoxidase system on primary normal human bronchial epithelial cells by inhibiting DUOX activity and exposing the cell interior to oxidative stress (24). These multiple deleterious *in vitro* effects of pyocyanin in inducing tissue damage and resisting host defense were studied and demonstrated *in vivo* by Lau and co-workers (2004) (25). These authors compared levels of infection and tissue damage in mice infected with wild type (WT) or pyocyanin-deficient strains of *P. aeruginosa* and reported that pyocyanin-deficient *P. aeruginosa* mutants produced less severe pneumonia compared to the lobar pneumonia, produced by wild type (WT) *P. aeruginosa* (strains PA14, PAO1). Pyocyanin-deficient *P. aeruginosa* mutant strains, when embedded in agar beads and used to infect mice intra-nasally, resulted in lower bacterial counts, compared to WT *P. aeruginosa*, demonstrating the role of pyocyanin in establishing chronic lung infection (Lau et al. 2004) (25). Production of pyocyanin increases when bacterial growth occurs in a biofilm pattern such as the chronic bacterial infection in CF (26).

Moreover, at the molecular and cellular level, pyocyanin induced CF-like effects, where it was found to inactivate vacuolar ATPase-dependent cystic fibrosis transmembrane conductance regulator (CFTR) expression and localization (27). These authors demonstrated that CFTR expression and trafficking *in vitro* in cultured monolayers of 16HBEo- cells, and *ex vivo* in primary nasal epithelial cells, are inhibited in a dose-dependent manner by pre-incubation of these cells with pyocyanin. Thus, compromising CFTR expression by pyocyanin may contribute to the aggravated oxidative stress, known to be associated with *P. aeruginosa* infection. This can be explained by the role of CFTR in mediating glutathione flux (28), which is compromised when CFTR expression and trafficking are abnormally decreased. This results in the consequent decrease of the antioxidant glutathione and worsening of oxidative stress.

Pyocyanin was also used by Caldwell and co-workers (29) to establish a chronic model of lung infection (repetitive intranasal exposure of mice to a physiologically relevant dose of pyocyanin, 25 µg/mouse, 3 times a week for 12 weeks). This caused neutrophilic infiltration, goblet cell hyperplasia, and pulmonary fibrosis.

2.1.1.3. Alginate:

Alginate is a component of the extracellular matrix around the bacterial micro-colonies (bacteria growing into biofilm), protecting them from phagocytes and complement activities (30). It is an acetylated polysaccharide composed of L-guluronic and D-mannuronic acids monomers (31). Muroid strains of *P. aeruginosa* overproduce alginate persistently over long periods unlike non-muroid strains which

produce less alginate (32). Acetylation of alginate increases the resistance of mucoid strains of *P. aeruginosa* to antibody-independent opsonic killing by phagocytes and confers resistance to the non-opsonic but alginate-specific antibodies, found in the sera of normal human and CF infected patients (33). Alginate production has been found to increase in a hypoxic environment such as the mucous layers in CF patients (34). Hypoxia may result from increased oxygen consumption by both bacteria and neutrophils, the latter needing oxygen to initiate a respiratory burst to kill bacteria (35).

2.1.1.4. Type III secretion system (TTSS):

P. aeruginosa encodes a secretory apparatus that consists of 15-20 membrane associated proteins, which deliver bacterial effectors directly into the host cell cytoplasm, allowing them to manipulate signaling and cellular processes in the host cells (36). Among such bacterial effector proteins is exo-enzyme S (ExoS), which is known to induce apoptosis of epithelial cells and macrophages (37, 38) and inhibit phagocytic activity of macrophages (39). Expression of the virulence factor ExoS increased and correlated with mortality in the first three days post- infection in an agar bead model of lung infection in rats (40). Other effector proteins include exo-enzyme T (Exo-T) and exo-enzyme U (Exo-U). ExoT shares 75% homology with Exo-S and has been reported to inactivate host cell proteins that maintain the actin cytoskeleton in Hela cells (41) Exo-U has phospholipase A activity and was reported to cause damage to the mammalian cell membrane and necrotic cell death (42)

2.1.1.5. Quorum sensing system:

This system coordinates expression of survival genes, genes encoding virulence factors and biofilm formation, among the entire bacterial population once it reaches a certain threshold of bacterial density or “quorum” (43, 44). This system works by bacteria-to-bacteria signaling where the bacterial cells produce low molecular weight signaling molecules, the extracellular concentration of which is related to the population of the producing organism. In gram negative bacteria, e.g., *P. aeruginosa*, these molecules are acyl homoserine lactones (AHLs) (45, 46). Two quorum sensing systems exist in *P. aeruginosa* (lasR-I and RhIR-I); each of which has a linked gene (R and I genes). When the extracellular signaling molecule, acyl homoserine lactone (3OC12-HSL) reaches a critical threshold concentration based on the density of the secreting bacterial cells, it interacts with LasR inside bacterial cells, where the resulting 3OC12-HSL-LasR complex activates the transcription of several bacterial genes encoding certain virulence factors (e.g., biofilm formation, inflammation induction, secretion of elastase, protease, rhamnolipid, and pyocyanin) (26, 47). This bacterial behavior indicates that *P. aeruginosa* coordinates its pathogenesis and function as a population, and not individually, inducing inflammation and biofilm formation, when the density of its population reaches a certain level. More importantly, this quorum sensing system was found to be, among other factors, contributing to the bacterial resistance to tobramycin, hydrogen peroxide and neutrophils, through blocking neutrophil activation *in vitro* (48). These authors reported that quorum sensing mutant strains of *P. aeruginosa*, unlike the wild type

strains, were more susceptible to hydrogen peroxide and phagocytosis by neutrophils *in vitro*.

2.1.1.6. Proteases:

P. aeruginosa secretes proteases, primarily elastase, which facilitate the invasion of host tissue (44). Other proteases include phospholipase, lecithinase and protease IV. Protease activity helps the bacteria to evade host defense mechanisms. Alkaline protease and elastase, secreted by *P. aeruginosa*, have been known to cleave immunoglobulins and inactivate complement, which compromises their role in host defense (12). Moreover, elastase and protease IV have been reported to cleave surfactant proteins A and D and consequently compromise their immune function (49).

2.2. Host factors in response to *P. aeruginosa*:

Host defense against infection is mediated by a variety of mechanisms that fall under two main components of the immune system: innate and adaptive immunity. Innate immunity includes anatomical barriers such as skin, hair, chemical barriers such as lysozymes and phospholipase in tears, wax in ears, and acute inflammation, which is activated once a pathogen or agent penetrate the body. This innate immune response is antigen-independent, immediate with a maximal response and results in edema and recruitment of phagocytic cells to the site of infection. Adaptive immune response is antigen-specific, with a lag period between invasion of the body by a pathogen and the maximum response (50, 51) . The main difference between innate and adaptive immune systems as reviewed (50, 51) is in their mechanisms and targets of recognition. The

adaptive immune system can recognize an almost unlimited creation of antigens using a somatically generated repertoire of highly specific antigen receptors on T lymphocytes and antibodies on B lymphocytes. This leads to subsequent activation and clonal expansion of the cells expressing the appropriate antigen-specific receptors and antibodies (50-52). The innate immune system relies on germ cell line-encoded receptors called pattern recognition receptors (PRRs), to identify a limited set of pathogen-associated molecular patterns (PAMPs), which are structural features of the pathogen (50-52). An example of PAMP (LPS) and the PRR that recognizes it (TLR-4) is discussed in the next section on epithelial cells.

This project dealt with responses in the first seven days following infection, with a particular focus on the first three days. With this focus on early events, we focused upon innate immune defenses in the studies within this thesis.

The innate immune system consists of several distinct modules that carry out different functions in host defense. These components are the surface epithelium, the phagocyte system, acute phase proteins, natural killer cells, mast cells, eosinophils, and basophils (50, 51). Clearance of *P. aeruginosa* from the airways takes place through defensive functions and innate immune responses, performed and coordinated by several cell types (44). We next summarize immune functions of the cell types that are considered to be important in innate immunity to *P. aeruginosa*:

2.2.1. Epithelial cells:

The respiratory epithelium is resistant to bacterial invasion via tight junctions (53) between cells and by the expression of mucin (54), which represents the machinery of mucociliary clearance. Epithelial cells also secrete antimicrobial peptides, e.g.,

defensin which is directly involved in host defense (55). Epithelial cells also have cell surface receptors that recognize and bind specific bacterial surface structures, e.g., pili of *P. aeruginosa* binds the asialoGM1 receptor on the epithelial surface, and the bacterial membrane component, lipopolysaccharide (LPS), binds toll-like receptor-4 (TLR-4) on epithelial cells, producing an inflammatory response and IL-8 production. Epithelial cells also express CFTR that binds to a ligand on LPS of *P. aeruginosa* (56, 57).

2.2.2. Phagocyte system (Immune cells):

Alveolar macrophages, the resident mononuclear cells in the lung, are the first line of defense against the microbes (44). Macrophages play an essential role in the early protective response to acute *P. aeruginosa* pneumonia. They phagocytose pathogens, synthesize and release reactive oxygen species (ROS) and release various inflammatory cytokines such as TNF- α , IL- β , IL-6 and IL-8 (44).

In addition, the neutrophilic response to biofilm bacteria is a major component of host defense against *P. aeruginosa*. The accumulation of activated neutrophils in the airways in the case of biofilm bacteria is part of the innate immune response to *P. aeruginosa* lung infection (48, 58-60). Neutrophils are short-lived with life span of 1-2 days and are equipped with many effector mechanisms. In contrast to macrophages and mast cells, neutrophils are not resident in peripheral tissue; rather they migrate from the circulation to the site of infection under the effect of cytokines and chemokines produced by resident macrophages, mast cells and epithelial cells (50, 51). This recruitment of neutrophils is dependent on production of the chemotactic

chemokine (IL-8) and the lipid inflammatory mediator, LTB₄. Sources of these neutrophil chemoattractants include macrophages, lung epithelial cells, endothelial cells and neutrophils (44).

Neutrophils contain three types of granules as reviewed by Segal and Witko-Sarsat et al. (61, 62). These are 1) the azurophilic (primary granules): containing myeloperoxidase (MPO), elastase, cathepsin G, and proteinase-3, and bactericidal/permeability increasing protein (BPI). 2) specific granules (secondary granules) containing lactoferrin and 3) Tertiary granules containing gelatinase. As for neutrophil killing of gram negative bacteria exemplified by *P. aeruginosa* (pathogen in our study), MPO is used to generate super oxide radical (respiratory burst), as detailed next; Elastase has been shown to directly degrade the outer membrane protein F of *P. aeruginosa*, known to be important in maintaining the integrity and morphogenesis of the bacterial cell membrane (63). Of note, mice deficient in neutrophil elastase are more susceptible to *P. aeruginosa* (64).

Neutrophils, like other phagocytes, engulf the pathogen and enclose it in a vacuole called (Phagosome) by a process known as phagocytosis that precedes killing of the microbe. Neutrophils then kill the vacuole-enclosed microbe by oxygen-dependent or oxygen independent mechanism (61, 62).

The oxygen-dependant mechanism is the process of killing which relies on oxygen consumption. Neutrophils synthesize superoxide radical (O_2^-) from molecular oxygen and nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), a process known as the respiratory burst. This begins with assembly of the proteins that form NADPH oxidase, which will use NADPH as an electron donor to couple

with molecular oxygen, converting it to superoxide anion; the latter is then dismutated to hydrogen peroxide (H_2O_2) by superoxide dismutase (65, 66). H_2O_2 reacts with myeloperoxidase enzyme (MPO), one of the main components of primary granules of neutrophils, in the phagosomal vacuole or in the extracellular space to form H_2O and hypochloric acid (HOCl). HOCl is known for its powerful bactericidal properties (65, 66). These reactions are described in Figure 1 below.

Chemical reactions of the respiratory burst and halide/myeloperoxidase system as reported by Lacy 2006 (65) and Quinn et al. 2004 (66).

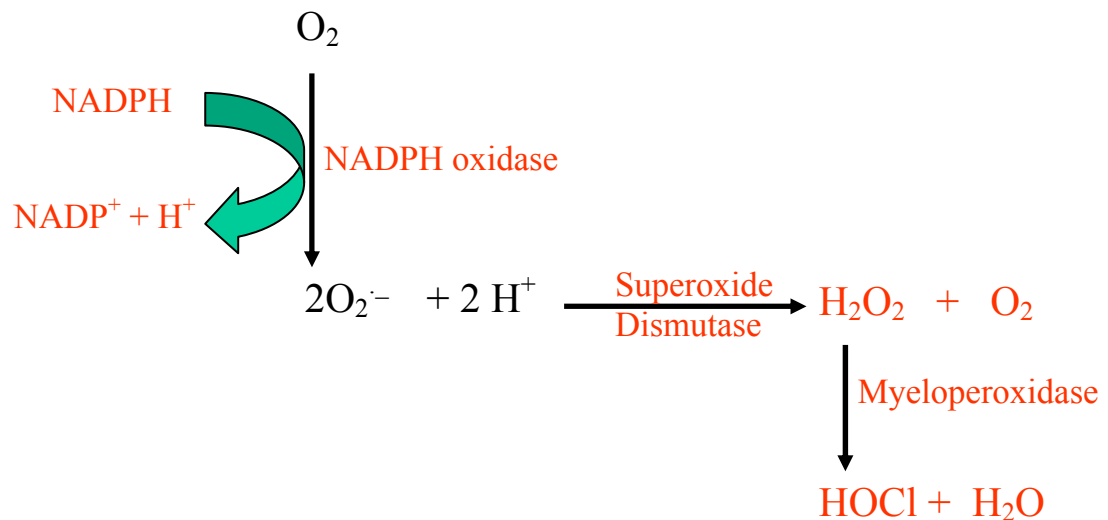


Figure 1.1: NADPH oxidase in the oxygen-dependent microbial killing pathway of neutrophils.

The active respiratory burst by the intensively recruited neutrophils in case of biofilm bacteria (chronic infection) causes accelerated oxygen depletion in the endobronchial mucus of infected CF patients (34). This will lead to anaerobic conditions in their

endobronchial mucus layer, a condition that was demonstrated to increase alginate secretion (34), thereby promoting biofilm formation and chronicity of the infection.

Oxygen-independent mechanisms include killing of the microbe independently from the respiratory burst and directly by BPI (67), cathepsin G (68), lactoferrin (69), defensin (70) and lysozyme (71), where the exact effector protein(s) and peptide(s) as well as the underlying mechanisms are pathogen-dependant.

2.3. Chronic lung infection with *P. aeruginosa* in obstructive lung diseases:

Among obstructive lung diseases, Cystic Fibrosis (CF) is the most frequently associated with chronic *P. aeruginosa* lung infection due to the dehydrated viscous mucus and consequent impaired mucociliary bacterial clearance. CF is an autosomal lung disease characterized by a mutation in the gene encoding CFTR protein, which results in dysfunction of this protein in airway epithelial cells, ducts of sweat glands, the pancreas, and the reproductive system. CFTR protein is a chloride conducting channel; its functions are: 1) outward conduction of chloride ion (72); 2) prevention of excessive sodium re-absorption by inhibiting the activity of epithelial sodium channel (ENaC) (27); 3) mediation of glutathione flux through epithelial cells (28); and 4) serving as a receptor for *P. aeruginosa*, as amino acids 108-117 on CFTR bind LPS of *P. aeruginosa* (57, 73, 74), resulting in a coordinated, rapid and self-limiting inflammatory response that quickly removes these bacteria from the respiratory tract (73, 74). According to the above first two CFTR functions, mutation and dysfunction of CFTR in CF patients leads to increased sodium re-absorption and chloride retention with consequent increase in water re-absorption by osmosis, thereby decreasing the

normal physiological volume of the airway surface liquid (ASL) and inducing a change in its components. This condition will lead to the transformation of the normal mucus to a dehydrated and viscous form that accumulates, forming mucus plugs, thereby impairing mucociliary clearance and increasing chances of entrapping bacteria in CF airways (57, 73, 74). In terms of the third CFTR function, glutathione flux is impaired in CF patients, with a consequent decrease in its airway level and hence its mucolytic effect on the mucus layer of the ASL is compromised (201). The decreased mucolytic and antioxidant effects of glutathione contribute to the increased viscosity of the mucus, resulting in failure of immediate bacterial clearance, and aggravation of oxidative stress, respectively. As to the fourth function, mutant CFTR fails to bind to *P. aeruginosa* LPS as would WT-CFTR in normal subjects (74) consequently; bacteria remain in the airway rather than being cleared efficiently. All together, the pathophysiological consequences of the four impaired functions of CFTR lead, in an integrative manner, towards the outcome of impaired bacterial clearance. The entrapped non-mucoid bacteria eventually mutates to a mucoid strain (30, 75, 76), able to form a biofilm, more resistant to host defense and to antibiotics, and can remain in the lungs of patients for years.

P. aeruginosa is the predominant pathogen to cause chronic infection in CF patients, infecting 80% of patients by late adolescence. In contrast, in other genetic diseases where loss of ciliary clearance occurs (e.g. immotile ciliary disorders), infection with this organism appears after 30 years of age and occurs only in 15% of cases (56). The predominance of *P. aeruginosa* as an etiologic agent of chronic infection in CF patients may be caused by the specific interaction between CFTR and

LPS. Using immuno-dot blots, Pier and co-workers visualized binding of biotinylated CFTR peptide 103-117 to *P. aeruginosa* or its outer core LPS but not the incomplete LPS. This was reviewed by Campodonico et al. (56) where impaired CFTR/*P. aeruginosa* binding and interaction in CF patients leads to an inability to clear infection as quickly as would occur in normal individuals with WT and sufficient expression of CFTR in their airway epithelia.

The mucoid strains of *P. aeruginosa*, which grow into micro-colonies within the biofilm, are characterized by antibiotic resistance. Among a variety of mechanisms behind this resistance, as reviewed by Hoiby (77), is the binding of the positively charged aminoglycoside antibiotics to the negatively charged alginate polymers, thereby reducing antibiotic diffusion (78); secretion of β -lactamase by *P. aeruginosa*, which cleaves β -lactam antibiotics (79); and a specific bacterial genetic determinant that links bacterial type VI secretion (t6s) to antibiotic resistance, thereby rendering the biofilm resistant to a subset of antibiotics (80, 81). Despite the fact that chronic infection can occur in both CF and non-CF patients, 90% of CF patients are infected with mucoid (alginate-producing) strains of *P. aeruginosa* (82, 83) as opposed to only 2% of non-CF patients (84, 85). The prevalence of mucoid strains of *P. aeruginosa* in CF patients can be attributed to the following etiological reasons: 1) the hypoxic mucus in CF was found to be an up-regulating factor of alginate production (34), alginate being the matrix for biofilm formation; 2) oxidative stress in the CF airways, which can be attributed to chronic inflammation, is a positive factor for inducing the mutation of non-mucoid to mucoid strains. In support of this notion, Mathee and co-workers (86), using a model of biofilm growth, reported that typical non-mucoid

strains of *P. aeruginosa* mutated and converted to the mucoid strain *in vitro* when treated with low levels of H_2O_2 , mimicking H_2O_2 released from neutrophils *in vivo*. Therefore, the two factors of hypoxic mucus and oxidative stress combined together can predispose non-mucoid *P. aeruginosa* to mutate and convert into mucoid strains, thus explaining the establishment of chronic lung infection in CF patients following their infection with non-mucoid strains of *P. aeruginosa*.

Other pathological conditions that may predispose the host to chronic infection include chronic obstructive pulmonary disease (COPD) (4, 5), bronchiectasis (4), and primary ciliary dyskinesia, an inherited disease (87). COPD is an umbrella term used to describe two different diseases, chronic bronchitis and emphysema; COPD patients may suffer from one or both of them. Chronic bronchitis is characterized by swollen bronchioles with inflammation that induces excessive mucus secretion and consequent clogging of the bronchioles, increasing the likelihood of entrapping microbes and subsequent development of a chronic infection. Emphysema is the loss of lung elasticity due to the permanent enlargement of the alveolar sacs, caused by destruction of the walls between the alveoli.

Cigarette smoking is the main cause of COPD; however, other predisposing factors include a genetic condition called α -1-anti-trypsin deficiency (AAT deficiency), environmental factors such as long term inhalation of dust or industrial fumes and toxins, and chronic infection in early childhood (88). One difference between COPD and CF patients is the occurrence of chronic infection later in life, unlike the early chronic infection seen in CF (5). Martinez-Solano et al (5), in their microbiological study on *P. aeruginosa* isolates from COPD patients, reported that

similar elements, known to characterize the chronic infection in CF, were also observed in the *P. aeruginosa* chronic infection in COPD patients. These common elements include the absence of evident inter-patient transmission, hyper-mutation of some bacterial strains, antibiotic resistance, biofilm production, and decreased motility (5).

Inflammatory cytokines in chronic lung infection with *P. aeruginosa*: Chronic intrapulmonary infection with *P. aeruginosa* is known to induce a sustained inflammatory response, characterized by massive neutrophil recruitment to the lung, increased pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6) and chemokines [macrophage inflammatory protein-2 (MIP-2) and keratinocyte chemo-attractant (KC)], mouse homologues of human IL-8, in broncho-alveolar lavage fluid (BALF) in mice studies (89). Increased levels of TNF- α , IL-6, and IL-8 in BAL of CF patients in response to *P. aeruginosa* infection are reported as well (90, 91).

2.4. Whey Protein:

Bovine whey proteins are a natural by-product of milk, derived during the cheese-making process and representing about 20% of total bovine milk protein (92). They are rich in all of the essential amino acids, with higher concentrations than vegetable proteins such as soy or wheat proteins (93), and are absorbed more efficiently compared to amino acid solutions (94). Upon removal of casein from whole milk during cheese-making, the remaining liquid, whey has a protein concentration of about 65%.

Whey proteins are separated and concentrated by one of two methods:

1) Cross-flow microfiltration: using filter membranes with molecular weight cut-off of 1 kDa, whey proteins are separated according to their molecular weight.

2) Ion exchange: whey proteins are separated based on their electrical charge.

Whey can be filtered to one fifth of its original volume to produce a whey protein concentrate (WPC), with a protein content of about 80%. It can be additionally microfiltered to raise its protein concentration to greater than 90%; a form of whey product called whey protein isolates (WPIs) in which non-protein components of whey are removed, such as lactose and cholesterol (13). We decided to use the cross-flow microfiltration-prepared whey protein due to the following advantages:

1. Denaturing of whey proteins is minimal
2. Proteins are separated without the use of heat or chemicals
3. Increased calcium and decreased sodium content
4. Better amino acid profile

(http://www.lef.org/prod_hp/abstracts/php-ab221.html)

In addition to the above-mentioned advantages of whey protein prepared by microfiltration, additional advantages were also reported when comparing the hyperbaric pressure-treated formula of whey protein, prepared by microfiltration with those prepared by ion-exchange. Pressurized whey protein that was originally prepared by microfiltration showed higher digestibility and tissue GSH inducing effects compared to that prepared by ion-exchange (Jing et al. 2001, Master dissertation, McGill University).

The potential effects of hyperbaric pressure treatment of whey protein are discussed in greater detail in the next chapter. However, the difference in responses depending on

the method used to prepare the whey isolate led us to use as a base whey product one that was prepared by microfiltration. However, a disadvantage of the micro-filtration method is the cost, which is twice that of whey prepared by the ion exchange method.

2.4.1. Components of whey protein Isolates (WPIs):

The components present in WPIs, used in this study as well as other aspects of analysis are listed in tables (2.1 – 2.5) below, as adapted from Inpro 90 certificate of analysis (Vitalus, Canada):

Table 2.1: Components of WPI supplied by Vitalus, Canada

Whey Protein Fraction:	% of Total Protein
β -Lactoglobulin (β - LG)	48%
α -Lactalbumin (α -LA)	18%
Bovine serum albumin (BSA)	2%
Immunoglobulins (Ig)	3%
Glycomacropeptide (GMP)	28%
Lactoferrin (LF)	< 1%

Table 2.2: Chemical analysis

Protein (dry basis)	92.5%
Lactose	0.4%
Moisture	3.6%
Fat	0.4%
Ash	3.0%

Table 2.3: Microbiological analysis

Standard Plate Count	< 5,000 CFUs/g
Coliform	< 10 CFUs/g
Yeast and Mold	< 100 CFUs/g
Salmonella	Negative in 375 g
Staphylococcus aureus	< 10 CFUs/g

Table 2.4: Amino acid (g/100 g protein)

Alanine	4.8
Arginine	2.0
Aspartic acid	10.8
Cystine	2.5
Glutamic acid	17.2
Glycine	1.5
Histidine	1.9
Isoleucine	6.6
Leucine	10.2
Lysine	8.0
Methionine	2.0
Phenylalanine	2.8
Proline	7.1
serine	4.8
Threonine	7.3
Tryptophan	1.7
Tyrosine	2.9
valine	5.7
Valine	5.7

Table 2.5: Minerals (per 100 g)

Calcium	0.5 g
Iron	0.8 mg
Magnesium	0.13 g
Manganese	< 0.05 mg
Phosphorous	0.3 g
Potassium	0.4 g
Selenium	< 0.04 mg
Sodium	0.2 g
Zinc	0.2 mg

β -Lactoglobulin (β -LG) constitutes about half of bovine whey proteins whereas human milk lacks β -LG. Moreover, β -LG is a rich source of essential and branched chain amino acids (BCAAs), in addition to biologically active peptides, which are inactive within the sequence of the native parent protein, and become activated upon release by gastrointestinal digestion or food processing (95). Further and interestingly, α -Lactalbumin (α -LA) also contains essential and BCAAs, which are important in enhancing muscle protein synthesis and injury repair (96). The whey components β -LG, α -LA and bovine serum albumin (BSA) contain the peptide γ -glutamylcysteine (20%, 25%, and 17.6% respectively) (97), which bypasses the rate-limiting step in glutathione (GSH) synthesis that is limited by feedback inhibition of the enzyme γ -glutamylcysteine synthase. Furthermore, β -LG and BSA contain the BCAAs leucine, isoleucine and valine. These indispensable amino acids, particularly leucine, are important factors in tissue growth and repair (98). Interestingly Biziulevicius and co-workers reported that oral administration of hydrolysates from β -LG, α -LA and BSA for mice for 5 days significantly increased the phagocytic ability of their peritoneal macrophages *ex vivo*. These authors reported this finding using different strains of gram negative and gram positive bacteria; in addition to fungi. Glycomacropeptides (GMP) are residues 106-169 of bovine κ -casein, which are released by chymosin hydrolysis during the cheese-making process. GMP is reported to be a potent immune enhancer; it significantly stimulated proliferation and phagocytic activity of the macrophage-like cell line U937 (18), and decreased host bacterial load in a murine model of sepsis(99) .

2.4.2. Antioxidant and immune-enhancing effects of native whey:

Several studies have demonstrated antioxidant protective and immune-enhancing effects of whey protein(s) in animals and humans, as well as *in vitro* studies. These studies are summarized in the following tables (2.2.1 – 2.2.6):

Table 2.6: Whey protein enhances humoral immune response in mice.

Authors	Subject of study & treatment	Challenge & outcome measures	Main findings
Bounous et al. 1989 (100)	C3H/HeJ mice were fed diets containing 20g/100g WPC, casein, or (casein+ cysteine), for 3 weeks	I.v. injection with sheep RBCs and study at day 5	Humoral immune response and splenic GSH increased in WPC-fed mice
Low et al. 2001 (101)	6-7 week-old BALB/c mice were fed WPC diet or chow for 84 days	- Oral immunization with cholera toxin and ovalbumin on days 0, 28 or 56 of feeding - Intestinal mucosal antibody response was studied at day 84 of the dietary regime	2-3 fold increase in mucosal antibody response to toxin and ovalbumin in the WPC-fed mice
Low et al. 2003 (102)	6-7 week-old BALB/c mice were fed a WPC-based diet or a non-milk protein-based diet for 12 weeks	-Immunization with a mixture of ovalbumin piolo, and cholera toxins were given orally at days 0 and 28 Flu and tetanus toxoid were injected s.c. at days 0 and 28 (flu); days 0 and 56 (tetanus). - Antigen-specific antibodies measured at weeks 0, 2, 4, 6, 8, 10, 12 in serum & intestine	-Both primary and secondary intestinal antibody responses were higher in WPC-fed mice -Secondary systemic antibody response in serum was higher in WPC-fed mice

Table 2.7: Supplementation with whey enhances both humoral and cell-mediated immune responses in mice.

Authors	Subject of study & treatment	Challenge & outcome measures	Main findings
Wong et al 2001 (103)	Female 8-10 week-old BALB/c mice were fed whey or soy bean (0.06 g/L) in drinking water or water alone for 6 weeks	I.p. immunization with ovalbumin at week 1, 3	Higher level of secondary anti-ovalbumin in sera of mice fed whey than those fed soy bean or water alone
Wong et al 2001 (103)	Female 8-10 week-old BALB/c mice fed with 20% whey or soy beans as the only source of protein	I.p. Immunization with ovalbumin at weeks 2 and 4	Both serum anti-ovalbumin at weeks 5, 6, and 7 and delayed type hypersensitivity were higher in the whey-fed group than soy bean-fed counterparts

From the studies, listed above in table 2.6, 2,7 one can clearly note that feeding with whey protein enhances humoral immune response following systemic challenge with sheep red blood cells (RBCs) in mice, in a glutathione-dependent manner, where the authors concluded that increased glutathione enhances lymphocyte proliferation (104). Enhancement of humoral immune response by whey protein concentrate (WPC) was also reproduced by other investigators who used different routes of antigen administration (Table 2.6, Low et al. 2001, 2003) where the mucosal antibody response, following oral immunization of mice with toxins (e.g. Cholera) was higher in WPC-fed mice compared to their chow-fed controls, persistently over the 84 days

of study. Further, the same authors demonstrated a similar enhancing effect of humoral immune response by feeding with WPC when they used another route of immunisation; they found an increased systemic secondary antibody response to subcutaneous immunisation with toxins, in the WPC-fed group compared with chow-fed controls. The authors attributed the above-mentioned enhanced intestinal humeral immune response by WPC to the activation of intestinal lymphatic tissue by WPC active component (lactoferrin) based on previous studies (105, 106). Although this conclusion was not tested directly in their study, their novel findings of enhanced intestinal humoral immune response by feeding with WPC potentiates the hypothesis that immune-enhancing effects of whey are not solely attributed to its pro-glutathione effect.

Enhancement of humoral immune responses by WPC was further confirmed by Wong and co-workers (studies listed in Table 2.7) who reported a systemic increased secondary antibody response following intra-peritoneal (i.p.) injection of ovalbumin. Moreover, these authors found enhanced cell-type mediated immune response in the whey-fed group compared to soy bean control. However, a universal limitation in all the above-reported studies (Table 2.6, 2.7) is the lack of cellular and molecular mechanisms underlying the whey-mediated immune-enhancing effect by whey protein, such transcription factors and cytokines, implicated in this beneficial effect.

Table 2.8: Whey protein increase longevity and protect against age-associated oxidative stress in mice

Authors	Subject of study & treatment	Challenge & outcome measures	Main findings
Bounous et al. 1989 (107)	B57BL/6 NIA mice were fed diets containing 20g/100 g whey, chow or casein between the ages of 21 to 27 months	Scoring Mortality (senescence)	Enhanced longevity (lower mortality) in the WPC-fed group
Bounous et al. 1989 (107)	B57BL/6 NIA mice were fed diets containing 20g/100 g whey, chow or casein between the ages of 17 & 20 months	Measurement of glutathione in the heart & liver	Increased heart and hepatic glutathione in the whey-fed group

As shown in table 2.8 whey protein enhanced longevity in mice compared to casein and chow (107); this was associated with increased glutathione levels in heart and liver in the whey-fed group, compared to casein and chow at the age of 20 months (designated as the age of senescence in mice). Of note, no difference in body weight gain or food intake over time of the study (6.3 months) among the three diet groups was observed despite the higher longevity in the whey-fed mice. This suggests that the protective effect of whey protein may not be explained by, or related to, differential nutritional values.

Table 2.9: Whey supplementation protects against ethanol-induced gastric ulcer in rats.

Authors	Subject of study & treatment	Challenge & outcome measures	Main findings
Rosaneli et al. 2002 (108)	Ratus norvergicus were fasting for 24 hrs, then given orally either 1g /kg of WPC in saline or saline alone at 10 ml/kg	-30 min. later rats were given orally absolute ethanol at 1 ml/kg -1 hr. following ethanol administration, animals were killed and stomach were cut and studied for ulcerative lesion	Significant partial inhibition of ulcerative lesion index in WPC-pre-treated mice compared to saline
Jahovic et al. 2005 (109)	Wistar albino rats were supplemented with 1 g of whey every 2 nd day for 2 weeks (2.5 ml of 40% whey in saline/rat), or saline alone (control) by oral gavage, while both groups were fed standard chow.	- On Day 15 rats were given ethanol (99%, at 5 ml/kg) to induce gastric ulcer or saline (control) orogastrically - 30 min. later gastric lipid per-oxidation and neutrophilic recruitment were assessed.	Lower hepatic and gastric lipid per-oxidation and neutrophilic recruitment in the whey supplemented group

Further, the antioxidant protective effect of whey protein was demonstrated in a direct oxidant injury (ethanol-induced gastric ulcer in rats), by Rosaneli et al 2002 (Table 2.9) (108) who reported a significant decrease in the ulcerative lesion index in the WPC-treated group compared to the saline-treated control. Although the authors studied one outcome measure only, (morphological assessment of ulcerative lesion index) without other redundant parameters or observed signs of oxidant-induced injury in their study, the assigned reference group (positive control group, given Carbenoxolone, a compound known to inhibit ulcer formation) is a strength of this study. The authors also reported a compromised inhibitory effect on ulcer formation by whey, when mice were pre-injected with bethionine sulfoximine, a specific inhibitor of glutathione synthesis; indicating that protective effect of whey is mediated, at least partially, by the pro-glutathione effect of whey (108).

Amelioration of ethanol-induced gastric ulcers by pre-treatment with whey protein was also reported by Jahovic and co-workers (Table 2.9) (109) who demonstrated significantly lower levels of both hepatic and gastric neutrophilic recruitment and lipid per-oxidation in the rats, given WPC orally prior to ethanol insult of their stomach, compared with saline-pre-treated controls. Although the conclusions of the above-mentioned two studies are similar in the sense that oral pre-treatment with whey protein infers protection against gastric ulcer, one can note that Rosaneli et al. gave rats a single oral dose of whey protein (about 0.4 g/rat), while Jahovic et al. gave rats whey for 14 days (1 g/rat, each other day) ahead of the insult with ethanol. This comparison suggests that protection can be afforded by short pre-insult supplementation.

Table 2.10: Supplementation of HIV-infected patients with whey protein increased glutathione level in different components of blood

Authors	Subject of study & treatment	Challenge & outcome measures	Main findings
Micke et al. 2001, 2002 (110), (111)	Double blind clinical trial. HIV patients were fed 45 g /day of WPI for 2 weeks or 6 months	Measurement of total GSH before and after treatment	Increased plasma concentrations of total GSH compared to baseline
Grey et al. 2003 (112)	Double-blind clinical trial with 24 CF patients	10 g of WPI or casein were given twice a day to stable patients for 3 months	Increased lymphocyte total GSH in WPI-fed patients without improvement in pulmonary function
Moerno et al. 2006 (113)	Double blind clinical trial with 18 HIV-infected children, give WPC for 4 months	Leukocyte count, Erythrocyte glutathione, and co-infection were observed	-Significantly higher level of erythrocyte glutathione and observed higher CD4/CD8 ratio and lower co-infection in WPC-treated patients

WPC supplementation to HIV patients resulted in elevation of glutathione in plasma, blood monocytes or RBCs (Table 2.10). Although these results are encouraging, yet no robust improvement in the main outcome measure (e.g. blood leukocyte and T-lymphocyte counts) was reported except a non-significant observed increase in CD4/CD8 T-lymphocytes ratio, associated with observed lower occurrence of co-infections over the period of study in patients fed with WPC as compared to the control patients (113). Further trails using of WPI, (which contains higher percentage of proteins, 90% vs. 80% in WPC) and/or using pressurized whey protein in HIV patients are warranted to test if better clear-cut immune enhancing effect can be achieved

Table 2.11: Whey as well as its components enhances innate immune functions of neutrophils.

Authors	Subject of study & treatment	Challenge & outcome measures	Main findings
Wong et al. 1997 (15)	Sheep neutrophils + LF, LP, (LF-LP), α -LA, BSA or WPC for 20 min. <i>ex vivo</i>	Measurement of chemo-tactic migration of neutrophils, as well as superoxide anion production	No effect was observed with any of the indicated components or whole whey protein (WPC) - Only (LF-LP) and WPC increased superoxide anion production in a dose-dependent manner - α -LA similarly increased superoxide anion production but the effect was not significant
Rusu et al. 2009 (14)	Human neutrophils incubated with a whey protein extract, α -LA, β -LG or GMP for 24 hrs <i>ex vivo</i>	Stimulation with fMLP and estimation of neutrophil functions	Increased superoxide anion production, neutrophil chemotaxis, degranulation and phagocytosis in a dose-dependent manner

The above-listed studies (Table 2.11) demonstrate that whey proteins also have enhanced effects on the immune defence system via increasing the initiation of the respiratory burst of neutrophils *ex vivo* (14, 15), where neutrophils are recognized as the first line of defence against microbes. According to Rusu et al. (14), whey has no direct effect on neutrophils; but primes them to respond more vigorously to a stimulus (e.g. fMLP). This priming effect of whey or its components is time-dependent, with an optimal effect after 24 hrs incubation with neutrophils. This may explain why no effect was observed on the chemotactic migration of neutrophils when they were pre-incubated with whey or its components for only 20 minutes (15), compared to the evident increase in the neutrophil chemotactic response after 24 hrs of pre-treatment (14). Although these results are encouraging, the beneficial effects of whey protein, in particular enhancing neutrophil immune functions, reported and discussed above, have to be tried in *in vivo* animal models of infection for further validation and consideration. Whey protein also enhanced the adaptive immune response in animal models using several routes of antigen administration. All together these studies demonstrate that whey protein has immune-enhancing and antioxidant effects. Although some of these effects, demonstrated to be successful in animal models of infection (Table 2.6 – 2.9) have not yet been tested in human subjects, the beneficial clinical effects of whey protein seen to date are encouraging (110, 111, 114, 115).

2.5. Pressurization of Whey Protein:

High hydrostatic pressure has advantages over the traditional thermal method of food processing; where the latter causes detrimental changes to the processed food,

such as degradation of vitamins by high temperature (116). Pressure processing permits the retention of food quality and impacts uniformly all parts of the whey solution, as compared to the more non-uniform effects exerted via the thermal method. High hydrostatic pressure, within 400-1000 MPa, has been used in several studies as a means to change the functional properties of food so as to improve its health benefits, without negatively affecting or compromising its nutritional value (117).

Amino acid profile of lyophilized pressurized whey protein powder *per se* (as the case for the native whey product, provided by the company certificate, page 22, 23) or from *in vivo* digestion are not available at the present time. However, free amino acid profile of filtered (< 1 KD cut-off) hydrolysates from pepsin and pancreatin-*in vitro* digestion of both native and pressurized whey proteins as reported by Vilela et al. hydrolysates (19).

2.5.1. Mechanisms of pressure-induced modification of whey protein:

Mechanistic aspects through which pressure leads to structural changes in proteins as reviewed by Boonyaratanakornkit et al. (2002) (118) Lopez-Fandino (119) and others (120-122) who studied the effect of pressure on whey proteins are summarized as follows:

Ionic interactions: Ion pairs between the negatively and positively charged amino acid side chains stabilize the tertiary and quaternary structure of the protein. Pressure promotes the disruption of ion pairs and therefore induces protein denaturation.

Hydration: Water affects protein conformation by filling the crevices between amino acids (118, 123). This serves to facilitate motion of the side chains and polypeptide backbone. Pressure increases conformational changes thereby providing pathways for water to penetrate into the interior of the protein and occupy internal volume, which was previously excluded from interaction with the solvent (118). As a consequence of the penetration of water into the protein interior, pressure is expected to cause conformational transitions that lead to unfolding (118). Thus a higher degree of solvation is reached in the unfolded protein as compared to the folded native protein (124). This difference in solvation between conformational states represents the fundamental basis for the effect of pressure on protein structure (124).

Hydrostatic pressure induces the formation of intermolecular hydrogen bonds while intramolecular bonds are decreased (118, 125, 126), thereby causing increased conformational fluctuation (118).

Increased exposure of peptide bonds to proteolytic enzymes: The above-mentioned conformational changes and unfolding lead to increased exposure of peptide bonds to proteolytic enzymes during the digestion of whey. For example, β -LG, a major component of whey protein, has most of its peptide bonds buried in the interior of this globular protein hidden from digestive enzymes. This explains the resistance of β -LG to peptic digestion (127), whereas hydrostatic pressure induced reversible and irreversible changes in its structure (121). Further, *in vitro* peptic digestion of pressurized whey was shown to be significantly more efficient than that of its native whey counterpart, as indicated by the higher amounts of released low molecular weight peptides (19).

Increased sulfhydryl/disulfide (SH/S-S) interchange: Hydrostatic pressure unfolds whey protein thereby exposing sulfhydryl groups that were buried in the native β -LG (122), and increasing SH/S-S interchange that will result in new sites for the digestive enzymes and consequently novel peptides (120). In support of this possibility, analysis of peptide and amino acid derivatives of hydrolysates from the *in vitro* digestion of native and pressurized WPI by Vilela et al. 2006 (19) also showed that arginosuccinate and sarcosine were higher in hydrolysates from native whey relative to pressurized whey; and γ -amino butyric acid and phosphoethanolamine were detected only in permeates from hydrolyzed pressurized whey but not native whey. These altered amino acid and peptide profiles can give rise to potentially novel immune-modulating peptides for an enhanced immune response.

2.5.2. Reported effects of pressurized whey on immune response and disease models:

To the best of our knowledge, there are limited reports available in the literature on the immune-modulating or antibacterial effects of pressurized whey protein using *in vivo* disease models or human subjects, particularly comparing pressurized vs. native whey, which is the main point of the current thesis studies.

The following tables (2.12, 2.13) summarize the reported antioxidant as well as other positive effects of pressurized whey in cell culture, animal and human studies:

Table 2.12: Pressurized whey enhanced anti-oxidant potential as compared to its native whey counterpart (both *in vitro* and *in vivo*) and compared to baseline in healthy subjects.

Authors	Subject of study & treatment	Challenge & outcome measures	Main findings
Hosseini- nia et al. 2001 (128)	Rats fed n.whey or p.whey for 17 or 35 days	Weight gain, hepatic total GSH, and blood lipid per- oxidation were measured	In p.whey group: increased weight gain and hepatic total GSH, decreased lipid per- oxidation in blood
Vilela et al. 2006 (19)	CF cell line	Incubation of cells with hydrolysates from: n.WPI or p.WPI for 48 hrs; intracellular GSH was measured	Intracellular total and reduced GSH were higher in cells incubated with hydrolysates from p.WPI vs. n.WPI
Zavorsky et al. 2007 (129)	18 healthy females and 18 healthy males	Orally given: 15, 30, 45 g/day for 14 days	Increase in total GSH was linear with the given amount of p.whey protein

Table 2.13: Pressurized whey improved lung function and attenuated the inflammatory response in CF patients, increased endurance to exercise, and improved quality of life in COPD patients.

Authors	Subject of study & treatment	Challenge & outcome measures	Main findings
Lands et al 2010 (130)	27 CF patients	Open-label study; p. whey was given orally at 20 g/day for < 18 year-old patients, and 40 g/day for older patients	Compared to baseline: enhanced nutritional status and improved forced expiratory volume in 1 second (FEV1), a pulmonary function test. PHA-stimulated IL-8 in cultured whole blood tended to decrease
Laviolette et al 2010 (131)	22 COPD patients	Oral supplementation with 20 g/day of p. whey or placebo casein for 16 weeks	In p. whey group: significant increase in cycling endurance test time (CET) & improved dyspnea

The above listed studies (Tables 2.12 and 2.13) demonstrate that pressurized whey protein has a glutathione enhancing effect, reflected in attenuated inflammatory responses *in vitro* (19) and *in vivo* (130) and increased antioxidant potential in an *in vivo* animal study (128). Further, pressurized whey protein improved nutritional status and lung function in CF patients (130) as well as exercise endurance in COPD patients (131). This may be due to the increased digestibility of whey and the consequent

alteration in peptide profile available for absorption as a result of pressurization. Thus, the altered peptide spectrum of the digested pressurized whey protein may have enhanced immune modulating effects. These effects, although in part mediated through an antioxidant mechanism, can also be mediated by the release of biologically active peptides that would be otherwise buried and not released from the digested native whey protein. Such peptides may function as ligands to specific receptors on the surface of inflammatory cells, thereby modifying one or more of the immune functions/responses. For example, the tri-peptide GLF (glycyl-leucyl-phenylalanine), derived from residues 51-53 of α -lactalbumin, has been shown to stimulate superoxide anion production by human neutrophils *ex vivo* (132).

Connecting Statement

Summary of the overall rationale

From the above reported studies, we can conclude that pressurization of whey proteins results in certain physicochemical changes (enhanced digestibility, altered peptide spectrum). Since digested whey components were found to enhance phagocytosis and bactericidal effects *ex vivo* to a higher degree compared to native counterparts (17, 133), then it is speculated that more efficient digestion resulting in potentially a novel absorbable peptide profile will result in a greater immune enhancing effect and help the host to resist and control infection at higher efficiency than native whey. Another contributing rationale to the above-mentioned hypothesized immune enhancing effect of pressurized whey is its antioxidant properties, which may protect neutrophils from oxidant-induced death. Hosseini-nia et al. (134) reported that the mortality of pressurized whey-fed, *P. aeruginosa*-infected mice was lower than that of casein-fed mice littermates. Yet, differences between the abilities of native and pressurized whey protein supplementation to enhance the host defense to infection are not known. The potential mechanisms of this hypothesized ability of pressurized whey to strengthen the host defense against infection are also unknown. Therefore the overall objective of this study was to prove that pressurized whey proteins enhance the host's ability to clear bacterial infection to a greater extent, compared to native whey.

For this overall objective, we set three specific aims:

- 1) To identify the time course of the inflammatory response, infection and protein oxidation in lung lavage of *P. aeruginosa*-infected and sterile beads-instilled control mice C57BL/6 mice (Chapter 4).
- 2) To study the inflammatory response and lung bacterial burden in mice pre-fed with native or pressurized whey protein and infected intra-tracheally with *P. aeruginosa*. This includes a preliminary study (Chapter 5.1), in which a third, casein-based diet group was designated, in addition to the main study (Chapter 5.2).
- 3) To study the potential mechanisms behind the expected attenuated inflammatory response and enhanced innate defense in the pressurized whey-fed group. The studied mechanisms are reported in Chapters 5 and 6.

CHAPTER 3

Animal model of Chronic Pulmonary Infection

With *Pseudomonas aeruginosa*

3.1. Summary:

In this chapter a theoretical background regarding the model of this study, an agar beads model, is addressed. The methodology section describes the preparation of the bacterial inoculums, infection method, necropsy, BAL fluid collection procedure and lung bacterial assessment. Finally, the clinical signs and parameters identifying this model are reported.

3.2. Background and history:

Cash and co-workers, in 1979 (135), were the first to originally devise an animal model mimicking chronic lung infection by embedding *P. aeruginosa* in agar beads before instillation intra-tracheally into the lungs of rats, where they observed that the bacterial population, recovered from the lungs within 3 days, was higher (10^6 CFUs) than the originally instilled bacteria (10^4 CFUs). Thereafter, they were able to detect bacteria in the lungs at a longer post-infection period, for up to 35 days. In 1987, Starke et al. (136) adapted this model to mice. The rationale behind embedding *P. aeruginosa* in agar beads is to physically impede removal of bacteria by the mucociliary beating, thus maintaining a persistent inflammatory

response, mimicking the chronic inflammation that occurs in CF. If the same inoculum is instilled in the lung as free swimming bacteria, the host would clear it within one day by innate immune defense mechanisms such as mucociliary clearance and alveolar macrophages; such a condition is defined as an acute lung infection. However, the host immune response that is studied in this model of *P. aeruginosa*-impregnated beads represents the resistance of the host to establishment of sustained infection with mucoid *P. aeruginosa* trapped in mucus, as is typically seen in CF patients with chronic infection. It does not represent an acute infection with planktonic non-mucus trapped bacteria. This model has been used by different groups to study immune responses and interventions (89, 137-145).

3.3. *P. aeruginosa* strain and preparation of the bacterial inoculums:

As indicated above, the bacterial strain used in this study was *P. aeruginosa* 508 (mucoid strain, originally extracted from a CF patient), showing a mucoid appearance of growth when plated on blood agar or tryptic soy broth agar plates. *P. aeruginosa* was loaded in agar beads as previously described (140). Briefly, an aliquot of 100 µl from frozen stock of *P. aeruginosa* was grown in 250 ml. of proteose peptone broth, placed on a shaking incubator at 37 °C overnight, following which an aliquot was further grown in 100 ml. of proteose peptone broth for 3.5 hrs under the same conditions to reach about 10⁹ CFUs/ml.. Bacteria were then concentrated by centrifugation at 11,000 rpm at 4°C and re-suspended in 2 ml. of PBS. A one ml. aliquot of this latter suspension was mixed with 9 ml. of sterile and warm agar (trypticase soy broth 3%, agar 1.5 %). This bacterial mixture with the

warm 9 ml. of agar (10 ml) was rapidly poured into 150 ml. of stirring mineral oil that was pre-warmed to 52°C. Stirring continued for 16 min. (the first 6 min. at room temperature; followed by another 10 min. under cooling with ice around the flask). This mixture was then centrifuged at 11,000 rpm at 4°C for 20 min. to precipitate the beads and separate them from the mineral oil. Beads were then transferred to new 15 ml. tubes, washed by spinning with PBS at 1500 rpm 4 times at room temperature, examined under the microscope to ensure the round shape and size (50 – 200 µm in diameter) and stored at 4°C until used within a week.

The day before infection, an aliquot of beads slurry (0.1 ml) was diluted (40 times by adding 3.9 ml. of PBS to the 0.1 ml. aliquot) and homogenized for 60 seconds, plated on agar plates in serial 10-fold dilutions, incubated overnight at 37°C and assessed the following day by counting CFUs on the plates to validate the inoculum strength as previously described (140). Based on this CFU count, an aliquot of the beads slurry was diluted with PBS to reach a concentration of 16×10^6 CFUs/ml. 50 µl of this diluted slurry contained 8×10^5 CFUs/mouse.

3.4. Animals and description of infection method:

C57BL/6 female mice weighing 16-18 g upon arrival (Charles River Laboratories, St. Constant, Quebec, Canada) were housed in individually ventilated cages, with 12 hrs light and 12 hrs dark cycles in a clean environment at the McIntyre animal facility, McGill University. They were given free access to water and fed a standard commercial chow diet *ad libitum* for 4-6 days to acclimatize to the new environment.

Mice were then weighed and instilled intra-tracheally with sterile agar beads as a control or agar beads loaded with *P. aeruginosa* (8×10^5 CFUs/mouse) using the non-surgical method of infection, adapted from Guilbault et al. 2005 (143), which can be summarized as follows:

- Mice were anaesthetized using a cocktail of ketamine and xylazine diluted in a sterile saline for a final concentration of 7.5 mg/ml. for ketamine 0.5 mg/ml. of xylazine. Mice were injected intra-peritoneally using this mixture at a dose of 20 mL/kg of body weight.
- Once the mouse was successfully anaesthetized with no pedal reflex, it was held in a restraining board in a supine position. The animal was held by its upper incisors using a thin clean thread (Figure 3.1).
- Immediately, the tongue was pulled aside by a sterile curved spatula and a curved 26-G gavage needle was then gently inserted and guided to the tracheal opening by visualizing the vibrating vocal cords, with the help of binocular surgical loupes (magnifying power, $x=4.6$) and trans-luminal light directed to the oral cavity of the animal. Once the blunt tip of the gavage needle entered the trachea, the inoculum (50 μ l containing 8×10^5 CFUs) was immediately instilled.
- Mice were then kept under heat (using a red lamp, distant about 3 feet from the cage for 45 – 60 minutes) to help the animals recover from anaesthesia.
- Upon regaining the righting reflex (awakening from anaesthesia) mice were put back in their cages.
- At Days 1, 3, or 7 post-infection, mice were by euthanized by CO₂ overdose using a CO₂ chamber and studied.

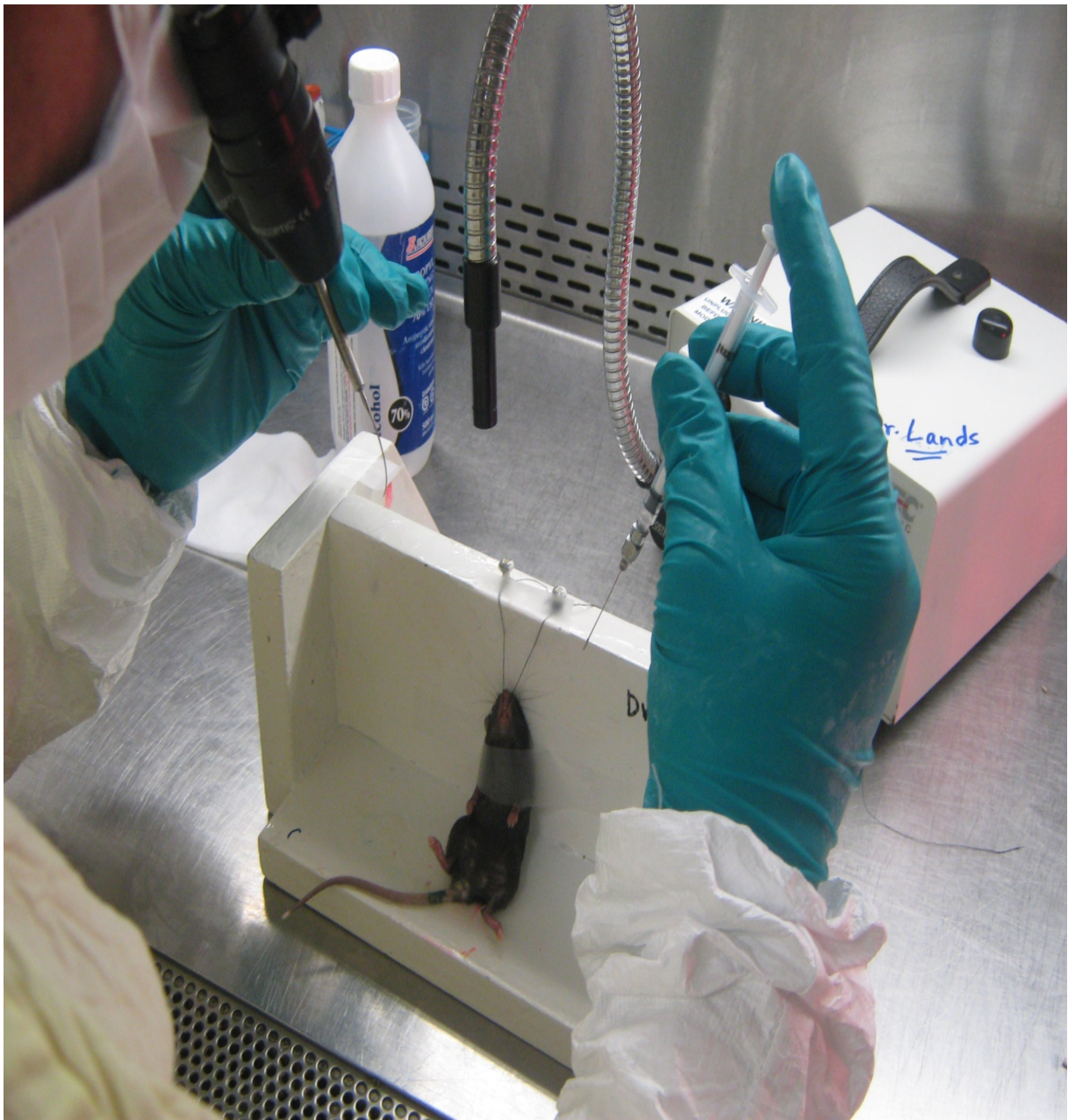


Figure 3.1: Mouse holding system and set-up for the direct visualization method of pulmonary infection

3.5. Procedure of broncho-alveolar lavage fluid and cell count:

The trachea of euthanized mice was exposed and lungs were lavaged with 2 ml. of sterile PBS, using a blunt needle. The volume of the retrieved lavage fluid was 1.4-1.8 ml. /mouse.

3.5.1. Isolation and total count of the inflammatory cells:

Lavage samples were then centrifuged for 10 min. at 2000 rpm. The cell pellet was re-suspended in a standard volume of 0.5 ml PBS. An aliquot of 25 μ l from each sample's cell suspension was diluted 1:1 with an equal volume of trypan blue, mixed and a drop from that mixture was used to count the total cells using haemocytometer. When necessary, a further dilution of the cell suspension was then made with PBS (e.g. 1:2) before diluting 25 μ l from the diluted cell suspension with trypan blue (1:1), prior to cell counting. In such cases, multiplication of the two dilutions was applied when calculating the final number of BAL cells/ml. The number of cells/ml. was then multiplied by 0.5 (standard volume of cell suspension, mentioned above) to express the cells/mouse BAL.

3.5.2. Differential cell count:

Based on the total cell count, another aliquot containing 50,000 cells (usually the volume of such aliquot ranged between 20 - 150 μ l) was diluted with PBS to

200 µl total volume, mixed to ensure homogeneity of cell distribution and spun at 1000 rpm for 5 min in a cytospin (each 100 µl was transferred into one pocket of the cytospin (2 slides were prepared for each sample, to have one as a backup). Slides were then dried for a few hours or overnight, and stained with Hema-3 stain (Fisher, cat. # 122-911). This set of Hema-3 stain consists of 3 separate chemical solutions, one is the fixative and the other two are Eosin and methylene blue stains designated as solution I, II, respectively. Set up for this staining requires pouring about 50 ml of each solution in a glass staining jar, with a fourth jar filled with tap water. Briefly, dried slides were dipped in the fixative for 20-30 seconds, tapped (from its side edge) on a paper towel to get rid of excessive fixative, and immediately dipped for about 30-40 seconds in solution I, II, and water jar, consecutively with tapping the slide from its edge on a paper towel between each 2 consecutive jars. A drop of paramount solution was poured on the stained cells, before a glass coverslip was gently put on them. Differential cell count was then done on these slides by counting 400 cells under 40 x magnifications. Supernatants of BAL fluid were immediately collected in small aliquots (120 µl each) and stored in -80°C until further protein and lipid peroxidation and total glutathione measurements.

3.6. Bacterial burden assessment:

Following the bronch-alveolar lavage procedure, whole lungs were weighed and immersed in 4 ml. of sterile PBS. Lungs were then homogenized for 60 seconds (homogenizer PT10135, Brinkman instruments Co., Mississauga, ON, Canada) under

cooling (by placing the 50 ml. tube containing the lungs and PBS in small a glass baker containing ice). Ten-fold serial dilutions of the lung homogenates were then made and plated on agar plates, incubated overnight at 37°C. CFUs/lung were counted, calculated the following day, and normalised per g lung tissue.

3.7. Clinical signs, lung bacteria and inflammatory response in a murine model of chronic lung infection with *P. aeruginosa*:

These features had been shown in several studies (89, 143) as well as in the present study (Chapter 4):

a) Body weight loss: Following infection, the host loses up to 10% of its weight daily in the first 3 days post-infection. By the 4th day post-infection, the host gradually starts to regain its baseline weight gradually. Most infected mice regain their baseline body weight by Day 7 post-infection (van Heeckeren et al. 2000) (89). This infection-associated weight loss was found to be attributed to a decrease in muscle mass secondary to systemic increases in inflammatory cytokines, particularly TNF- α and interleukin-1 β (IL-1 β) (89). Infection-associated muscle wasting can also be further explained by an increased need to synthesize proteins, necessary for the inflammatory response, e.g., acute phase proteins (APPs), which are rich in aromatic amino acids (146). Since muscle protein is the major source of these amino acids and APP levels increase 2-100-fold during inflammation, it can be assumed that for 1 g of synthesized APP, 1.5-2 g of muscle protein needs to be degraded to release an adequate amount of these aromatic amino acids (146). In addition to muscle wasting, another contributing explanation to body weight loss

can be anorexia, reducing the caloric intake of the host. Anorexia is a consequence of the inflammatory response (147). In this regard, IL-1 β , a pro-inflammatory cytokine, reported to rise systemically during infection (89, 147), causes anorexia (148). Another inflammatory cytokine implicated in the mechanisms of infection-associated body weight loss is TNF- α , which is known to induce cachexia and muscle wasting during the first 3 days post-infection with *P. aeruginosa* (89).

b) Lung bacteria: Although neutrophils are theoretically recruited to clear bacteria from the lung, one of the characteristic features of this model is the detection of bacteria in the lungs at any time point up to one week or longer (138, 141, 143, 149), due to the resistant nature of bacteria growing in a biofilm. In this regard, some authors showed that the lung bacterial burden is higher at Day 2 post-infection compared to Day 7 (40); others (89, 150) showed that it peaks at day 3 post-infection.

c) Neutrophil-dominated pulmonary inflammatory response: Several authors (89, 138) have shown that inflammatory cells in the BAL fluid increase significantly in *P. aeruginosa*-infected mice compared to sterile bead-instilled controls at Day 2 or 3 post-infection; these inflammatory cells were shown to be about 90% neutrophils. This massive neutrophil recruitment to the lung is not due to impregnating the bacteria in the agar beads, but rather to the quorum sensing system, which activates bacterial virulence factors that induce inflammation, at certain cell density. This conclusion was reached by Lesprit and co-workers (151) who used the agar beads model with a mutant virulent strain of *P. aeruginosa* lacking the signaling molecule that coordinates the expression of virulence factors, which activate epithelial secretion of IL-8. A macrophage response was elicited in that study instead of the

known neutrophil-dominated response when the wild type *P. aeruginosa* (with functioning virulence factors) is used. This indicates that the expression of the virulence gene (*lasR*), rather than LPS of *P. aeruginosa* or the agar beads, is critically important in eliciting the sustained neutrophil-dominated inflammatory response to clear the infection. The pro-inflammatory cytokines (TNF- α , IL-1 β , and IL-6) and chemokines MIP-2 and KC, (mouse homologues of IL-8) in BAL fluid have also been reported to increase in this model of *P. aeruginosa* lung infection (89).

d) Myeloperoxidase enzyme activity in lung tissue: Myeloperoxidase, which has been extensively used by authors as a marker of neutrophil recruitment to the lungs, was detected in lung homogenates and found to be several-fold higher in the infected mice compared to the non-infected controls, most-clearly in the first 3 days post-infection (138).

3.8. Relevance of this model to cystic fibrosis:

Although we did not use CF mutant or knock out mice in the model of this study of prolonged pulmonary infection, it is still relevant to the correspondent chronic infection often seen in CF patients, due to the following considerations and limitations:

1. The nature of the artificial manipulation used in this model, to load bacteria of a mucoid strain in agar beads result in holding the bacteria against the mucociliary clearance of the airways; this pathophysiological condition mimics the entrapment of bacteria in the mucus in CF patients.

2. CF knockout mice have intestinal fibrosis, consequently they have digestive difficulties and undergo significant mortality that would be a challenging drawback in such study, or otherwise costly intestinal-corrected CFTR mutant mice would be the alternative. In the meantime using a liquid Peptamin® as a dietary solution to the digestion problems will not be applicable for the current study, as the protein source in Peptamin® is whey, the studied intervention.
3. Bacterial virulence factors such as pyocyanin have been reported to down regulate CFTR expression in airway epithelial cells; this finding, if applicable *in vivo*, would imply a CF-like action in wild type mice from exposure to pyocyanin. This possibility, at last theoretically, increases the overlap and simulation between this model and the chronic bacterial infection seen in CF patients.

Appendix to Chapter 3

Survival and bacterial dose determination:

We considered 10^6 CFUs/mouse to be used for infection in our study based on Guilbault et al. 2005 who chose this dose based on several trials using different doses. However this dose of 10^6 CFUs /mouse in our model resulted in a survival rate of 71.4% (15 out of 21 mice survived the 3rd Day post-infection), whereas none of the mice, that were instilled with sterile-beads died, assuring the safety of the procedure. *P. aeruginosa* at 10^6 CFUs induced 11.6-fold increase in total leukocytes in BALF at Day 3 with neutrophils constituting 85.2 ± 1.7 % and macrophages $13.9 \pm$ % vs. 1 ± 0.5 % neutrophils and $97.2 \pm 0.5\%$ macrophages in BALF of sterilized beads instilled controls. However, the high rate of mortality when using 10^6 CFUs/mouse was concerning and needed resolution before going further in the study. This high rate of mortality apparently was due to the fact that our mice are females only, which are more susceptible to infection and inflammation with *P. aeruginosa* than males (142, 143). This justification is in accordance with the observation of Guilbault and co-workers who reported increasing rates of mortality in females compared to their male littermates, where they were obliged to show some representative results from males only due to this limitation (143). Therefore the above-mentioned survival rate of 71.4% associated with using 10^6 CFUs/mouse in females is not surprising. We then decided to try 5×10^5 CFUs/mouse. All the mice survived the infection when the dose was halved to 5×10^5 CFUs but the inflammation was mild and may not be the appropriate one to study our intervention.

To be conservative and avoid the drawback of undesired high mortality, in the meantime targeting intense inflammation and infection, we decided to try an intermediate bacterial infecting dose of 8×10^5 CFUS/mouse. Using this intermediate dose, 92.3% of the infected mice (12 out of 13) survived the 3rd Day post-infection; again with no mortality in the sterile bead control group. The intense inflammation and infection induced by using this intermediate dose were evident as reported in the next chapter (time course study) and as exemplified by values of lung bacterial and body weight loss in Table 3.1, below. This table is not presented to show any investigative question, comparison or time course, rather it illustrates examples of lung bacterial burden values and demonstrate that lung bacterial burden varies from occasion to another, even when using the same inoculum.

Table 3.1: Representative values of lung bacterial burden and body weight loss at different time points using various levels of bacterial inoculums.

Serial number	Time point & Bacterial dose of infection CFUs/mouse	Lung bacteria Median log CFUs/g tissue	Body weight loss as % of base Mean $\pm$ S.D.	n
1.	6 hrs 1×10^6	7.5	N/A	5
2.	24 hrs 1×10^6	6.28	- 4.3% $\pm$ 5.9	4
3.	24 hrs 5×10^5	5.3	- 5% $\pm$ 5.9	3
4.	12 hrs 8×10^5	6.08	N/A	3
5.	3 Days 8×10^5	9.2	- 19.5% $\pm$ 1.3	3
6.	3 Days 8×10^5	5.8	- 21.1% $\pm$ 2.7	4
7.	3 Days 8×10^5	5.6	- 11.3 $\pm$ 8.5	3

Each batch represents an occasion of infection that was run on a group of 2-5 mice in the same day.

CHAPTER 4

Time Course of Inflammatory Response, Infection and Oxidation of Broncho-alveolar Proteins in a Murine Model of Chronic Lung Infection with *Pseudomonas aeruginosa*

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Manuscript from this chapter is in preparation for submission to Immunobiology.

4.1. Connecting Statement:

Following establishment of the model and its reproducibility of infection and inflammatory response, we then evaluated the inflammatory response and infection at different time points (Day 1, 3, 7 post-infection). Since the enhanced antioxidant defence of pressurized whey is one of the potential mechanistic pathways explaining its anti-inflammatory and/or anti-bacterial effects, we measured lung protein and lipid oxidation in this time course study. Results from these outcome measures led to further investigations into protein and lipid oxidation in whey-fed mice in subsequent parts of the project (i.e. chapter 5).

4.2. Summary:

Lung infection with *P. aeruginosa* causes oxidative stress, reflected in increased oxidation products in BAL fluid. This study was undertaken to investigate the time course of protein and lipid peroxidation in the airways of mice with chronic intrapulmonary *P. aeruginosa* infection. **Methods:** Female C57BL/6 mice were infected with 8×10^5 CFUs of *P. aeruginosa* embedded in agar beads to simulate bacteria entrapped in thick mucus, using intra-tracheal instillation by direct visualization. Animals were studied at Day 1, 3, and 7 post-infection to determine lung bacterial burden and inflammatory response (weight loss, cells in bronchoalveolar lavage (BAL) and percentage neutrophils), protein and lipid peroxidation and BAL as well as total glutathione. **Results:** There was significant weight loss at Days 1 and 3, which was not fully recovered by Day 7. The inflammatory cells in BAL as well as the percentage of neutrophils were

significantly higher in the infected group at Days 1, 3, and 7. Lung bacterial burden was also significantly higher at Days 1 and 3, but was not significantly different from control at Day 7. Protein carbonyls as an index of oxidative stress were significantly higher in the BAL of infected mice at Days 1, 3, and 7. Unlike protein oxidation, lipid peroxidation in BAL fluid was not different between the groups at any of the time points. Total glutathione in BAL fluid tended to be lower in the infected group at the three time points. **Conclusion:** In a chronic lung infection model, protein oxidation in the airways is prominent early on, without concomitant lipid peroxidation, and persists despite waning infection and inflammation.

4.3. Introduction:

Patients with Cystic Fibrosis (CF) (1-3, 6), Chronic Obstructive Pulmonary Disease (COPD) (5, 6) or bronchiectasis (4) can become chronically infected with *Pseudomonas aeruginosa* (*P. aeruginosa*). This infection causes exuberant oxidative stress in the airways via a variety of mechanisms (7-10). This may render biologically important proteins dysfunctional (152). Bronchoalveolar lavage (BAL) protein carbonyls have been used as a reliable marker of protein oxidative damage in CF patients (153, 154). However, these studies did not elucidate the relation between infection and protein oxidation as not all patients were infected. Therefore the present animal study was designed to describe the time course of infection, inflammation, and airway protein and lipid oxidation in a murine model of prolonged infection with *P. aeruginosa*. Protein oxidation occurred early and persisted despite the waning of

infection and inflammatory infiltrate. However, there was no evidence of airway lipid per-oxidation.

4.4. Materials and methods:

4.4.1. Preparation of bacterial inoculums and infection method:

The animal model described in chapter 3 was used. Inoculums of 8×10^5 CFUs / 50 μ l were instilled intra-tracheally using a direct visualization non-invasive method as reported previously (143). For more details, see chapter 3.

4.4.2. BAL procedure and lung bacterial burden assessment:

At the indicated time points (Days 1, 3, or 7 post-infection) mice were terminated, BAL procedure and lung bacterial burden were performed as described in detail in chapter 3.

4.4.3. Measurements and assays:

4.4.3.1. Myeloperoxidase (MPO) enzyme activity:

MPO activity was measured according to Klebanoff et al. (155). Briefly, 20 μ L of lung homogenate or BAL supernatant were mixed with 980 μ L of MPO working solution, which was freshly prepared by mixing 107.6 mL H₂O, 12 mL 0.1 M sodium phosphate buffer (pH 7.0), 0.192 mL guaiacol, and 0.4 mL 0.1 M H₂O₂. The formation of tetra-guaiacol was measured spectrophotometrically at 470 nm, and the change in optical density was monitored for 5-6 min. MPO activity was calculated from the formula: enzyme units/mL = Δ OD/min. x 45.1 and expressed as enzyme U/g tissue.

4.4.3.2. Bacterial burden assessment:

Following the BAL procedure, the left lung was consistently excised and immersed in 3 mL of sterile PBS. Lungs were then homogenized and bacterial burden assessment was obtained as described in chapter 3.

4.4.3.3. Measurement of protein and lipid peroxidation:

BAL and tissue protein oxidation (protein carbonyls) were measured using an ELISA-based kit (Catalog. STA-310, CellBiolabs, Inc., San Diego, CA). BAL and tissue lipid peroxidation (HNE-Histone adducts) were measured using an ELISA-based kit (Catalog. STA-334, CellBiolabs, Inc., San Diego, CA). Both kits were used according to the manufacturer's instructions. ELISA-based methods are trusted and reliable quantitatively, unlike the semi-quantitative approaches such as western blot assay. They also allow the handling of more samples per work day.

4.4.3.4. Total glutathione in BAL:

Total glutathione in lavage supernatant was measured using an enzymatic method (114, 156). BAL supernatant was freshly deproteinized by adding and mixing 20 μ L of chilled 5% trichloroacetic acid (TCA) with 180 μ L of BAL supernatant from each sample. The mixture was spun at 12,000 rpm, 4°C for 10 min and stored at -80°C until later measurement. Protein-free supernatants were neutralized with 100 mM sodium phosphate buffer (pH 7.4; 5 mM EDTA) and pipetted into a temperature-controlled (37°C) cuvette. The assay reagents (dithiobis-nitrobenzoic acid [DTNB], NADPH and glutathione reductase) were thereafter added sequentially. Optical density was read kinetically at 412 nm. Glutathione concentration in the sample was calculated using a serially diluted glutathione standard curve.

4.5. Statistical analysis:

Data are expressed as mean $\pm$ SE. Student t-test was used to compare between sterile control and infected groups at Day 1, or 3 or 7 post-infection. Two-way ANOVA was used to compare between control and infected groups along the three different time points. One-way ANOVA followed by Tukey's post-hoc test were used to compare findings within the same group along the three time points of the study. Statistical significance was set at $p < 0.05$. Statistical analyses were conducted using Sigma Stat 3.02 software.

4.6. Results:

4.6.1. Body weight loss:

Mice instilled with sterile beads regained their weight by Day 3 (Figure 4.1). Mice infected with *P. aeruginosa*-loaded beads lost a significantly greater percentage of their pre-infection body weight compared to sterile bead-instilled mice amongst the groups ($p=0.001$). Weight loss in the sterile bead control group during the seven days post-instillation was not significantly different ($p=0.12$) unlike the infected group, where weight loss peaked at Day 3, and was statistically different over the seven days ($p=0.007$). Weight loss at Day 7 was significantly lower compared to Day 2, 3 of the same infected group ($p=0.019$, $p=0.04$, respectively) but was still trending to be higher than the weight loss of the sterile beads control at the same time (Day 7) ($p=0.06$). All together, this indicates a significant waning of the inflammatory response at Day 7 although sustained inflammation was still observed compared to the baseline.

4.6.2. Lung Bacterial burden:

Log CFUs in infected mice was significantly greater along the three time points compared to the sterile beads control ($p < 0.05$, Figure 4.2). Bacterial burden at Days 1 and 3 was significantly higher in the infected mice compared to the sterile beads controls (Day 1: $p < 0.01$, Day 3 $p < 0.05$). Bacterial burden decreased in both groups by Day 7 and was no longer statistically different between the groups. Bacterial readings were occasionally observed in sterile beads control because mice were housed in a clean environment and not in a pathogen-specific sterile environment.

4.6.3. Inflammatory cells in BAL:

P. aeruginosa infection induced a several-fold increase in the total number of inflammatory cells in BAL at Day 1. This increase was maintained significantly higher than the correspondent values in the sterile bead control group throughout the whole observation period (Figure 4.3A), although it waned by Day 7 post-infection. Differential count of the recruited cells in BAL from infected mice showed that 80-90% of the recruited cells were neutrophils at Days 1 and 3, and 50% at Day 7 (Figure 4.3B).

4.6.4. Myeloperoxidase (MPO) activity in lung tissue:

MPO activity as an index of neutrophil recruitment and activation was significantly higher in the infected group at Days 1 and 3 post-infection (Figure 4.4). At Day 7, MPO activity still tended to be higher in the infected group than the control of the same day (sterile beads).

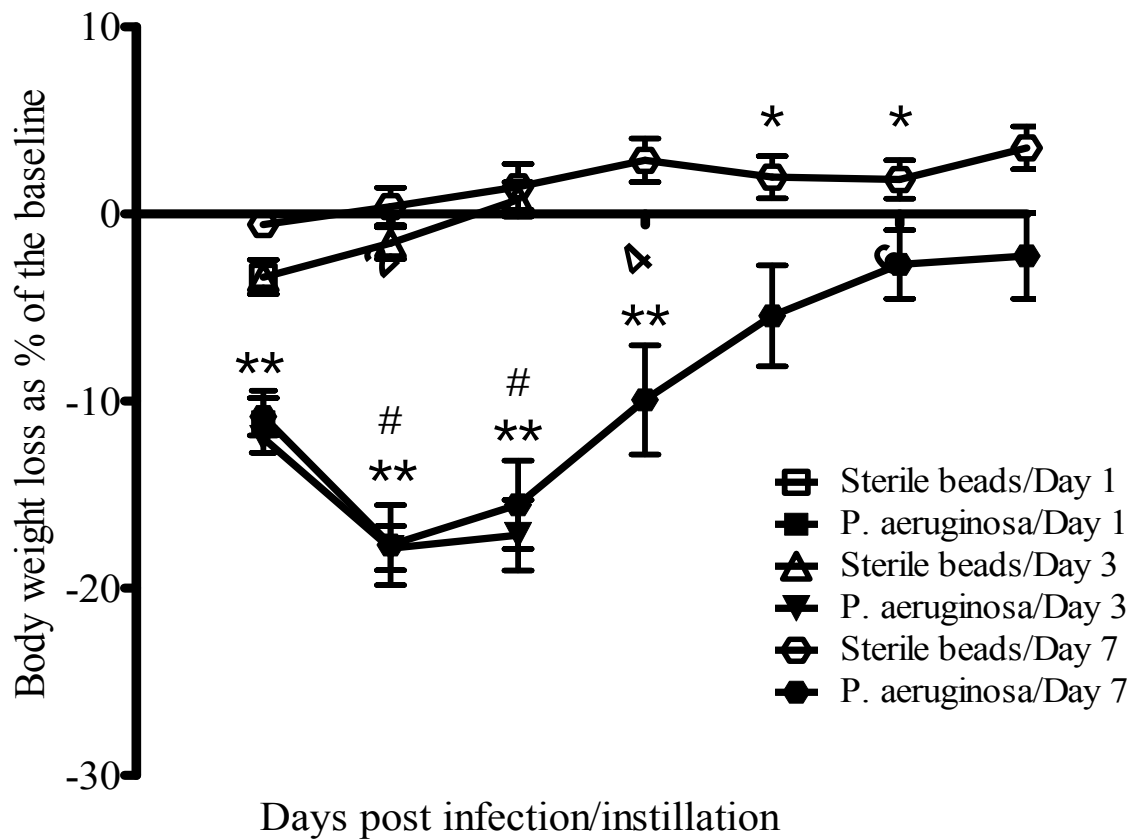


Figure 4.1: Animal body weights were scored daily for the experimental groups terminated at Day 1, 3, 7 post-instillation with sterile beads or post-infection with *P. aeruginosa*-loaded beads. Body weight losses as % of pre-infection weight were presented as mean $\pm$ SE. The upper two line-plots represent sterile bead control (Day 3: empty triangles, day 7 : empty hexodes). The lower two line-plots represent infected groups (Day 3: inverted solid triangle, Day 7: solid hexodes).

P. aeruginosa-infected mice lost a significantly higher percentage of their body weight as compared to their sterile bead-instilled littermates at Days 1-6 post-infection (** $p < 0.001$, * $p < 0.05$ vs. sterile bead control on the same day). # $p < 0.05$ vs. Day 7 of the same infected group. $n = 7-8$ (Day 1 group), $n = 10-14$ (Day 3, 7 groups).

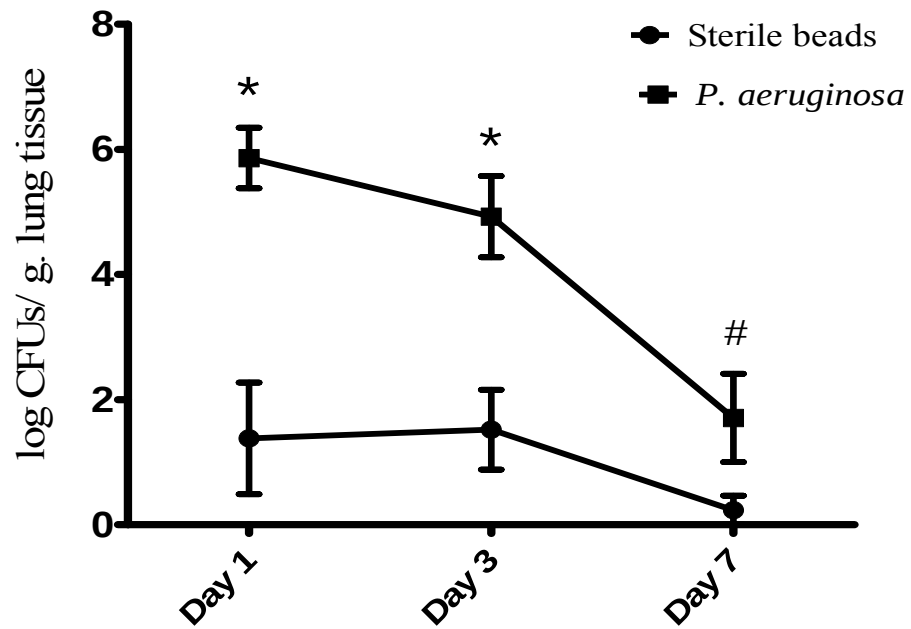


Figure 4.2: Lung bacterial burden expressed as mean $\pm$ SE. Solid circles represent sterile bead control values; solid squares represent *P. aeruginosa* infected mice. * $p < 0.05$ significantly higher in infected animals compared to their sterile bead controls on the same day. At Day 7, bacterial burden was significantly lower than at days 1 and 3 in the infected group; # $p < 0.05$, $n=7-8$ (Day 1), 10-14 (Day 3, 7).

Figure 4.3.A:

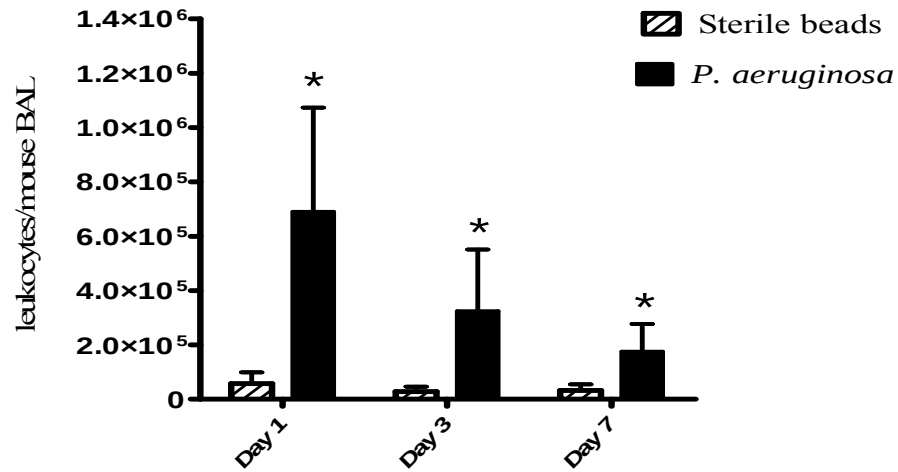


Figure 4.3.B:

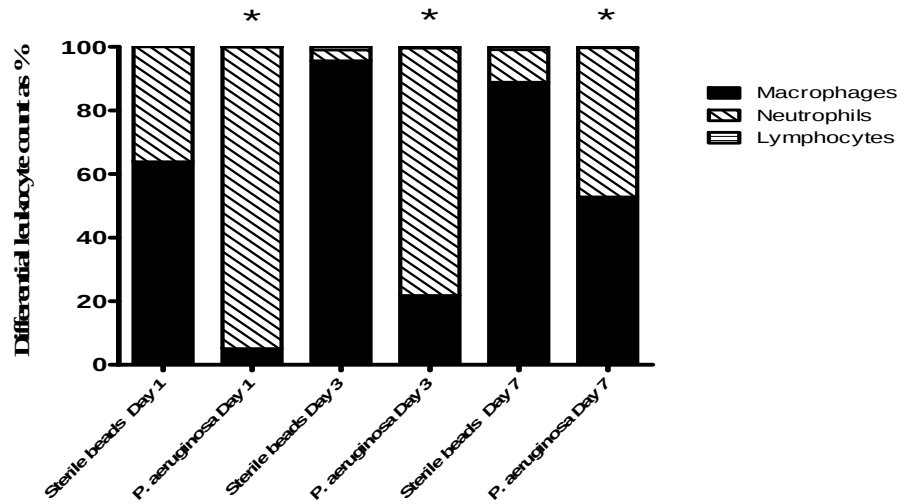


Figure 4.3: Total leukocyte count in BAL of sterile bead controls (hatched bars) and *P. aeruginosa*-infected mice (solid bars) at Days 1, 3, and 7, expressed as mean $\pm$ SE, $n = 7-9$ (Day 1), $8-12$ (Days 3, 7). (A) Differential count of the inflammatory cells in BAL, $n = 3-5$ (Day 1), $5-9$ (Days 3, 7). (B) * $p < 0.05$ vs. sterile bead control on the same day.

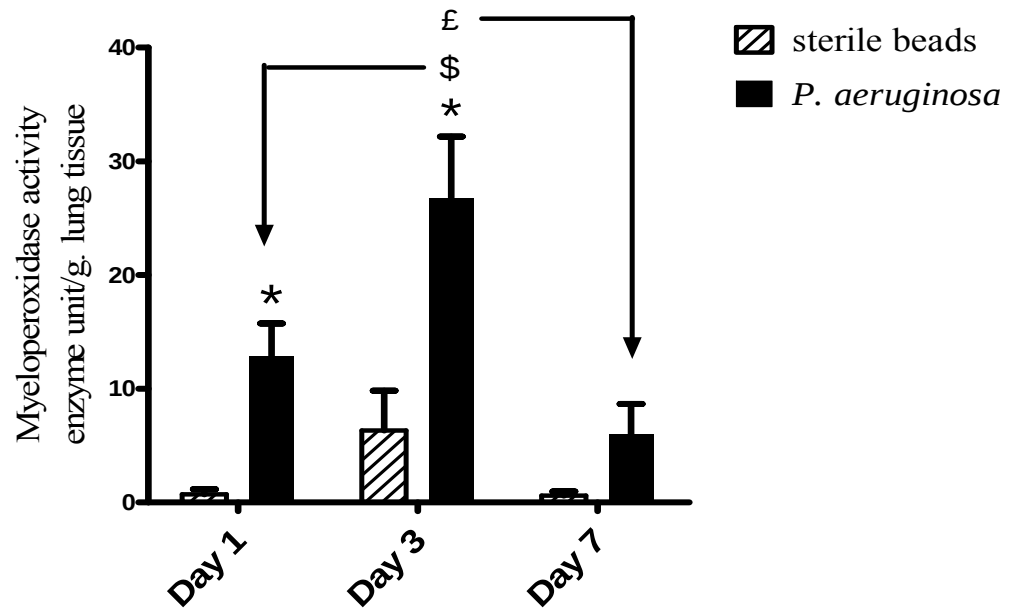


Figure 4.4: MPO activity in lung tissue homogenates presented as mean $\pm$ SE. *P. aeruginosa* induced a significant increase in lung MPO activity at Days 1 and 3 (solid bars) compared to their control littermates (hatched bars). * $p < 0.05$ vs. sterile bead control of the same day; \$ $p < 0.05$ vs. Day 1, *P. aeruginosa*-infected animals; £ $p < 0.05$ vs. Day 3, *P. aeruginosa*-infected animals, $n = 7-8$ (Day 1), $10-12$ (Day 3, 7).

4.6.5. Markers of oxidative stress:

Protein oxidation: BAL protein carbonyls were significantly higher in the *P.aeruginosa* infected group at all three time points (Figure 4.5). In contrast, lung tissue protein carbonyls, measured at Days 1 and 3, were not different between the sterile bead control and *P. aeruginosa*-infected groups (data not shown). However, at Day 7, lung tissue protein carbonyls tended to be higher in the infected group (mean $\pm$ SE: 1.58 ± 1.1 versus 5.8 ± 0.9 nmol/mg protein, sterile bead control versus infected groups, $n=2,3$, $p=0.08$).

Lipid per-oxidation: There was no significant difference in BAL or tissue lipid per-oxidation between *P. aeruginosa*-infected and sterile bead control groups at any of the time points (data not shown).

4.6.6. Total glutathione:

Kinetics of BAL glutathione levels were essentially comparable between the animals instilled with sterile beads or *P. aeruginosa*-laden beads along the three days post-infection (Figure 4.6). Glutathione levels tended to be lower in the infected group across time ($p=0.07$).

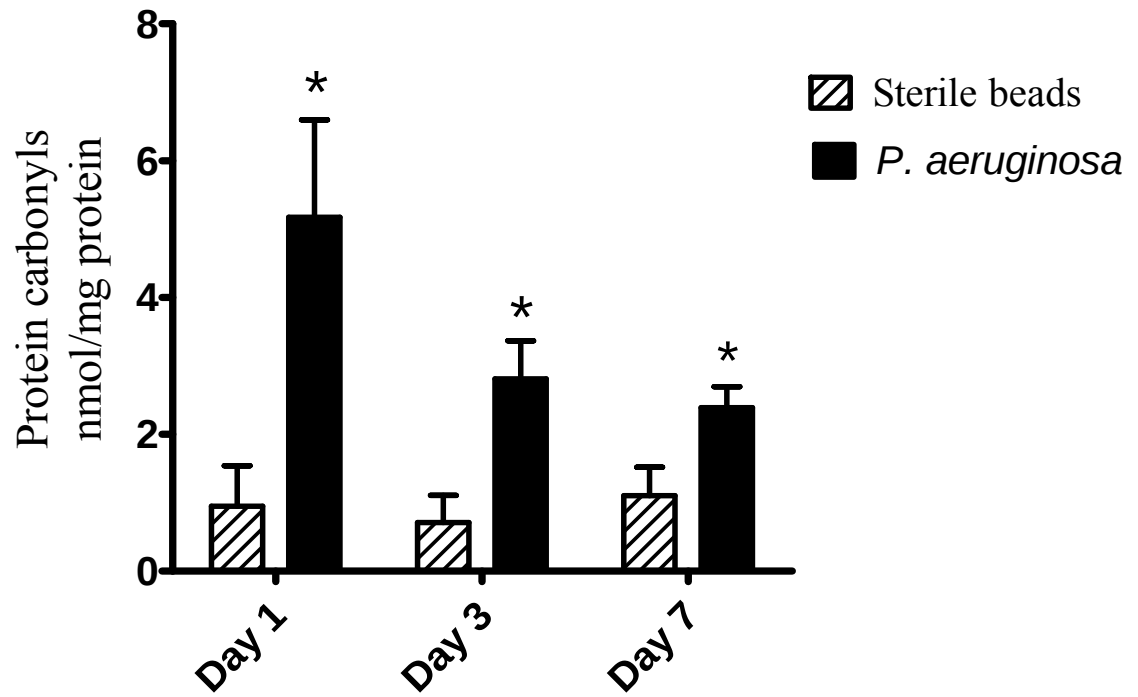


Figure 4.5: Protein carbonyls in BAL supernatants of sterile beads control (hatched bars) and *P. aeruginosa* infected animals (solid bars) presented as mean $\pm$ SE. * $p < 0.05$ compared to control animals on the same day, $n=7-8$ (Day 1), $7-10$ (Day 3, 7).

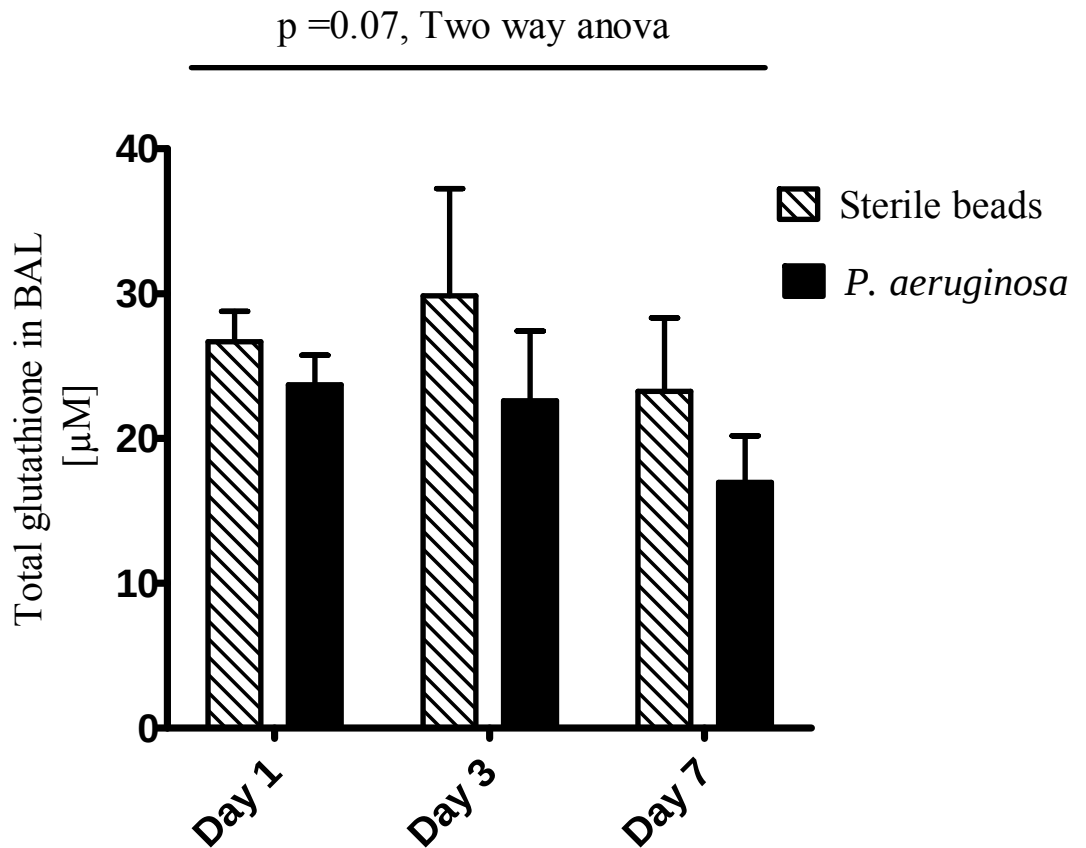


Figure 4.6: Total glutathione in BAL fluid supernatants of sterile bead instilled control (hatched bars) and *P. aeruginosa* infected mice (solid bars) presented as mean $\pm$ SE. Total glutathione tended to be lower in the infected groups compared to the sterile bead controls across the time points, $n=6-8$ (Day 1), 7-10 (Day 3, 7).

4.7. Discussion:

Although the time course of infection with *P. aeruginosa* in agar bead murine models has been previously reported (138, 140, 142), this is the first report to study the interaction of infection, inflammation, and oxidative damage beyond three days post-infection (143). We note that although lung bacterial burden and airway inflammation significantly diminished by Day 7, there is still ongoing airway oxidative stress. Further, the oxidative damage seems to be directed at airway proteins, and not lipids.

As previously reported (143), direct tracheal instillation of infected beads results in less inflammation and enhanced bacterial clearance compared to instillation via a tracheal incision. Thus typically, a higher bacterial inoculum is required in this model to produce the same level of inflammation as seen in models using an incision. Using weight loss as a marker of the systemic inflammatory response (89), we note a weight loss in the present study with an inoculum of 8×10^5 CFUs comparable to those studies with an incision using an inoculum of 2×10^4 to 10^5 CFUs (89, 142, 157). However, looking at the local inflammatory response, there was a smaller airway lumen neutrophilic infiltrate at Day 3 in the present study compared to an incision using 2×10^4 CFUs (0.5×10^6 in the present study vs. 2.2×10^6 in Day et al., 2004). Likewise when a higher dose of 10^6 CFUs/mouse was used trans-tracheally by Divangahi et al. 2004 (138), total inflammatory cells in BAL at Day 2 was 3.5×10^6 /mouse BAL. However, Divangahi and co-workers reported difficulty and poor tolerance by the animals with this higher dose at Day 7. When Divangahi and co-

workers used a lower dose of 2×10^5 CFUs/mouse, BAL cells at Day 2 were 1.6×10^6 and diminished at Day 7 to a level comparable to the total BAL cells at Day 7 in the present study. However, unlike the present study, they found no significant difference in BAL total and absolute neutrophil cell counts at Day 7. This suggests that the non-surgical method of infection with higher inoculums results in less local inflammation at earlier time points (Day 2 or 3) but more sustained inflammation and better animal well-being at later time points (Day 7).

We found an increase in airway lumen protein oxidation in the infected groups at all three time points. This was not reflected in tissue protein oxidation at Days 1 and 3, although tissue protein oxidation tended to be higher in the infected group at Day 7. We did not, however, see any evidence of lipid peroxidation. There was also a trend to lower airway total glutathione concentrations. Using a tracheal incision model with 2×10^4 CFUs in male C57Bl/6 mice, Day and colleagues found that infection at Day 3 increased airway glutathione concentrations but did not change tissue lipid and DNA oxidation markers (157). Further, Range and co-workers (158) saw no change in plasma markers of protein and lipid oxidation in response to treatment of pulmonary infection in CF patients. These and the current study's results suggest that the oxidative stress observed in the airway is not well-reflected in tissue or plasma, and thus should be looked for directly in airway fluid. Potential reasons for the lower glutathione concentrations in the infected group in the present study and higher concentrations in the study of Day and co-workers include differences in infection model (intra-tracheal in the present study versus trans-tracheal in Day and co-workers) and differences in gender (female in the present study versus male in the Day and co-

workers study). Female mice are more susceptible to inflammation and infection with *P. aeruginosa* (142, 143).

The present results suggest that therapies aimed at enhancing antioxidant protection in the airway lumen could be beneficial. Several attempts have been made to increase thiol and glutathione levels in the lung and lung lining fluid via different routes (159-165). Aerosolized GSH in CF patients improved lung function without reducing airway neutrophils or markers of oxidative stress (166, 167).

Low molecular weight substances (glutathione and lipids, our data and Day et al., 2004) appear not to be the primary target of oxidation during *P. aeruginosa* infection and substantial subsequent neutrophil-dominated inflammation. Rather, proteins are oxidized the most during these processes. Importantly, the observed oxidative protein modifications are irreversible, i.e., the proteins cannot be rescued. If these are the proteins essential for anti-bacterial defences, their inactivation may facilitate chronic bacterial colonization in susceptible patients, such as patients with CF. Importantly, low molecular weight antioxidants (i.e., inhaled glutathione) do not offer sufficient protection from protein oxidation (Griese et al., 2004) which necessitates the search for more effective antioxidant measures aimed at preventing irreversible protein oxidation. Oral treatment with N-acetyl cysteine (NAC) increased whole blood and neutrophil GSH and decreased airway neutrophils, IL-8, and sputum elastase in CF patients. However, there was no improvement in lung function or bacterial load (168). A recent one-month pilot study of supplementation with pressurized whey, rich in cysteine and branched chain amino acids, in CF children and adults, improved lung function in children, nutritional status in both children and

adults, and tended to decrease inflammatory markers, including PHA-stimulated interleukin-8 production in whole blood (115). Further, a one month feeding of C57BL/C mice with pressurized whey protein decreased lung bacterial load and protein oxidation at Day 3 post-infection (Kishta et al. 2011, submitted manuscript).

In summary, infection with *P. aeruginosa* results in an intense inflammatory response with associated weight loss and airway luminal infiltration. The inflammatory response is accompanied by significant oxidant damage to airway proteins, which is still evident at the time of waning infection and inflammation. This oxidation may affect protein function. Oral therapies aimed at limiting oxidant protein damage may be beneficial in helping to limit injury and lung bacterial growth resulting from *P. aeruginosa* infection. Animal studies as well as clinical trials aimed at testing the efficacy of antioxidant interventions should include airway protein carbonyls along with airway inflammation and infection as outcome measures.

CHAPTER 5

Pressurized Whey Protein Enhances Bacterial Clearance to a Higher Level as Compared to its Native Whey Counterpart

The manuscript from this chapter is in preparation for submission

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5.1. Connecting statement:

Since we found prolonged protein oxidation in the face of waning infection and inflammation in the time course study, we expected that pressurized whey, through its antioxidant potential would abrogate this oxidative stress with consequent anti-inflammatory and/or anti-bacterial effects. We then proceeded to test the main hypothesis of this study as to whether supplementation with pressurized whey has a superior effect in enhancing the bacterial clearance relative to native whey. Since our time course study showed also that lung bacterial burden and inflammation (weight loss, BAL cells, and lung myeloperoxidase activity) start to diminish after Day 3, we studied these primary outcomes in infected mice, pre-fed with different whey-based diets (native or pressurized) at Day 3 post-infection.

5.2. Summary:

Female C57BL/6 mice were randomized to semi-purified diets containing casein, native or pressurized whey as a protein source for four weeks before they were infected with 8×10^5 CFUs/mouse of *P. aeruginosa*, loaded in agar beads, and studied at Day3. Lung bacterial burden tended to be lower in the pressurized whey and casein groups compared to the native whey group. Two supplementary groups were then fed with native or pressurized whey-based diets for four weeks, infected and studied at Day 3 as described earlier. Analysis of whey-based experiments (native & pressurized) showed that lung bacterial burden was significantly lower by 11-fold in the pressurized whey group compared to the native whey group at Day 3. Potential mechanistic aspects to explain the enhanced bacterial clearance in the pressurized whey-fed group were then

investigated. Bactericidal activity and superoxide anion production of neutrophils at Day 1 were found to be higher in the pressurized whey group relative to their corresponding levels in the native whey group. Lower GM-CSF concentrations and protein oxidation were evident in the airways of pressurized whey group at Day 3; tendencies towards less MIP-2 (at Day 1, 3) and less IL-1 β (at Day 1) were both evident in airways of the pressurized whey-fed group. Conclusion: supplementation with pressurized whey protein can enhance the host's ability to clear bacterial infection, while reducing protein oxidation.

5.3. Introduction:

Studies showed that *P. aeruginosa* infection causes oxidative stress via a variety of mechanisms; this pathogen-induced oxidant damage was evident and mirrored in our time course study as persistent protein oxidation, along with waning but sustained infection and inflammation. Whey protein components as well as whey-derived peptides were reported to augment the innate defense functions of neutrophils (14) (15, 18, 169) and provide protection in animal models of infection (132). Permeates from the digested whey components, α -lactalbumin (α -LA) and β -lactoglobulin (β -LG), decreased bacterial metabolic activity *in vitro* to a greater extent compared to their native non-hydrolyzed α -LA and β -LG.

Exposure of whey proteins to hyperbaric pressure unfolds the proteins thereby increasing their antioxidant potential and digestibility, leading to an altered spectrum of peptides available for systemic absorption (19) with potential immune-modulating effects. Hence we hypothesize that pressurization enhances the antimicrobial effect of whey

protein. In this chapter we describe the experiments undertaken to prove this hypothesis using a feeding protocol with whey-based diets (native or pressurized) and a murine model of lung infection with *P. aeruginosa*, described in chapter 3. Upon finding more efficient bacterial clearance in the pressurized whey group, we conducted a series of experiments to explore the potential mechanisms behind this effect.

5.4. Preliminary study:

We conducted a preliminary study that aimed at evaluating and comparing the inflammatory response and lung bacterial burden in mice pre-fed with native or pressurized whey protein, along with a third diet group of casein pre-fed mice. Casein contains biologically active peptides (e.g. β - and κ -casein fractions), which can modulate the immune response (16, 169, 170). The immune-modulating effects of casein can enhance or depress innate immunity depending on the purity of the protein and the condition of testing (170). Injecting peptides from casein into mice protected them from *Klebsiella pneumonia* (171). We assigned a group of mice pre-fed with casein here as a non-whey control. Whey protein has been reported to have higher antioxidant defense against aging and enhanced longevity compared to casein in mice studies. Moreover, whey contains biologically active components (β -LG, α -LA, glycomacropeptide, etc.), which are known to enhance neutrophil function *ex vivo* (14, 15, 171). Therefore, whey is expected to perform better than casein in our model. However, the preliminary study showed that bacterial clearance and some of the anti-inflammatory effects of both casein and pressurized whey were superior to native whey (Table 5.1).

We decided to not continue with casein since the preliminary study showed that pressurized whey resulted in a greater bacterial clearance than native whey. As casein's effects on innate immunity have not been consistently positive (172, 173) we decided to continue our study with native and pressurized whey only.

5.4.1. Experimental design of the preliminary study:

Female C57BL/6 mice were randomized to casein as a non-whey control, or to native or pressurized whey protein-based diets. Mice were randomized to receive semi-purified diets containing casein, native or pressurized whey protein as the only source of protein for 4 weeks, and then infected intra-tracheally with 8×10^5 CFUs/mouse as detailed in chapter 3. We decided to use female C57BL/6 mice since they are more susceptible to pulmonary inflammation and infection with *P. aeruginosa* than males (136,137); this is expected to make the effect of our intervention more discernable.

5.4.2. Feeding protocol:

C57BL/6 female mice weighing 16-18 g upon arrival (Charles River Laboratories, St. Constant, Quebec, Canada) were housed in individually ventilated cages, with 12 h light and 12 h dark cycles in a clean environment at the McIntyre animal facility, McGill University. They were given free access to water and fed a standard commercial chow diet *ad libitum* for 4-6 d to acclimatize to the new environment. At the end of the acclimatization period, mice were weighed and randomized to their respective test dietary treatments, which consisted of semi-purified diets containing casein, native or pressurized whey protein isolate (WPI) as the only source of protein. The WPI used was Inpro 90 WPI

purchased from Vitalus (Abbotsford, BC). Its composition on a dry weight basis consisted of: protein > 92%; β -lactoglobulin 43-48%, glycomacropeptides 24-28%; α -lactalbumin 14-18%, bovine serum albumin 1-2%; immunoglobulins 1-3%, lactoferrin <1%. WPI powder was dissolved in doubly distilled water (15% w/v) and exposed to a single cycle of pressure of 550 MPa with a holding time of 1 min, using an Avure High Processing system model QFP 215L-600 (Avure Technologies, Columbus, OH). This was followed by lyophilization by spreading the solution of pressurized WPI on a metal container at -40°C for 5 d (Agriculture and Agri-Food Canada, St-Hyacinth, Quebec, Canada). The lyophilized pressurized or native WPI was incorporated at 20% (w/w) into isocaloric semi-purified diets (AING-93, MP Biomedicals, LLC, Santa Ana, CA). The diet composition consisted of (w/w): 36.9% cornstarch, 10% dextran corn starch, 9% sucrose, 14% fats, 5% alphacel non-nutritive bulk. The diets were stored at -20°C until later use for feeding. Food consumption was ascertained by daily monitoring the weight of leftover food and replacing it with fresh food. Mice were fed for four weeks during which time normal growth was assured by scoring their body weight every other day. They were found to gain 18-20 % of their baseline (pre-feeding) body weight. Daily food consumption per mouse was not different amongst the diet groups (Table 5.1). Studies were conducted in a staggered manner with several repeated experiments so that 1-3 mice were randomly allocated per diet group for each four-week feeding experiment.

Table 5.1: Mice daily food consumption (gram/day/mouse)

Number of mice & amount of food/cage	Casein-based diet	Native whey-based diet	Pressurized whey-based diet	Statistical significance
1 mouse/cage 10 g food/cage	2.94 $\pm$ 0.05	3 $\pm$ 0.07	2.92 $\pm$ 0.05	N.S.
2 mice/cage 20 g food/cage	2.55 $\pm$ 0.04 ¶	2.58 $\pm$ 0.03 ¶	2.55 $\pm$ 0.04 ¶	N.S.
2 mice/cage 14 g food/cage	N.A	2.83 $\pm$ 0.03 ¶	2.62 $\pm$ 0.05 ¶	N.S.

¶: Relative food consumption

5.4.3. Preparation of bacterial inoculum and infection protocol:

Mice were then infected with 8×10^5 CFUs/mouse using the non-invasive direct visualization method to inoculate *P.aeruginosa*-impregnated agar beads via the trachea directly into the lung, as reported by Guilbault et al. 2005 (143) and described in details in Chapter 3 of this thesis. The infected mice were then terminated at Day 3 post-infection as in previous mouse studies as well as our own time course study (Chapter 4), which showed that bacterial burden either peaks at or begins to diminish three days post-infection (89, 174, 175), and that the inflammatory response peaks at 3 days post-infection (89). Inflammatory cells were isolated from BAL fluid where total and differential counts were performed. Myeloperoxidase enzyme activity in lung tissue homogenates as an indirect index of neutrophil recruitment to the lung, and lung bacterial burden were assessed as described in details in chapter 4. Supernatants of BAL samples were collected and frozen for further analysis.

5.4.4. Broncho-alveolar lavage (BAL) procedure and assessment of lung bacterial

load: The procedure described in chapter 3 was used.

5.4.5. Results of the preliminary study:

Daily body weight loss was not different amongst the groups over the observed three days post-infection (Figure 5.1). Bacterial burden tended to be higher in the native whey group (table 5.1). Myeloperoxidase enzyme activity in lung tissue was significantly higher in the native whey group (table 5.1). Likewise, BAL fluid total

cell count was numerically but not statistically greater in the native whey group compared to the other two groups. Differential cell count in BAL fluid showed a neutrophil-dominated pattern without a notable difference amongst the groups (table 5.1).

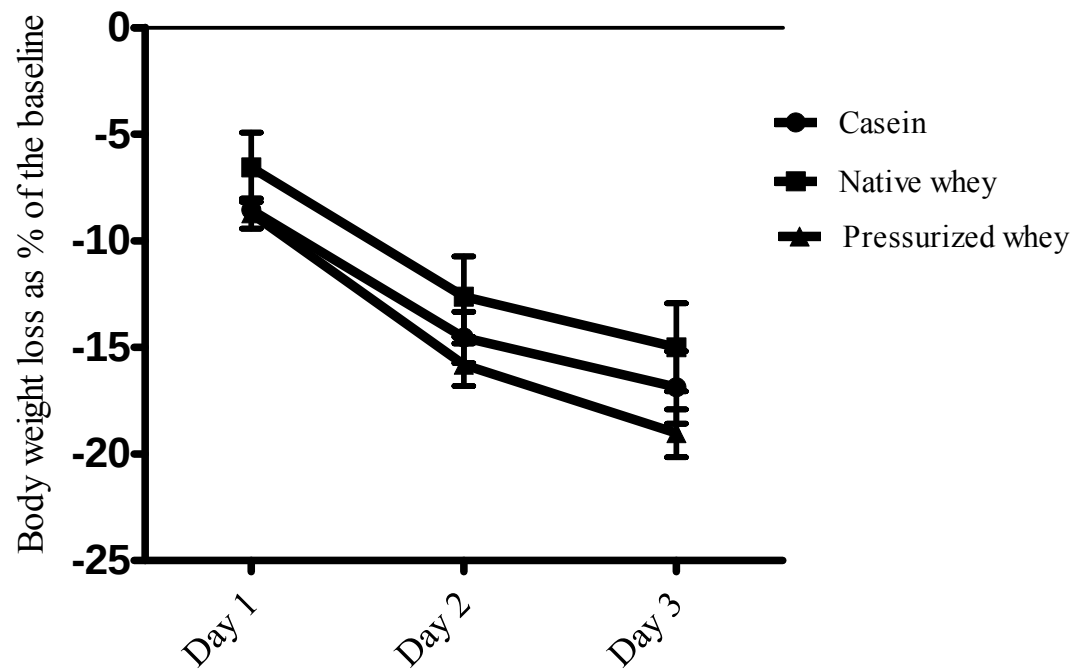


Figure 5.1: Daily body weight loss at Days 1, 2, and 3 post-infection in the three groups pre-fed with casein, native or pressurized whey-based diets for 4 weeks before intra-tracheal infection with *P. aeruginosa*. N=8.

Table 5.2: Parameters of inflammatory response and lung bacterial burden in the preliminary study are listed.

Parameter at Day 3 post-infection	Casein	Native whey	Pressurized whey	P value
Lung bacterial burden: mean log CFUs/g lung tissue	6.69 ±0.5	8.33 ±0.5	6.67 ±0.5	0.06
Total inflammatory cells in BAL	5±1.3 x 10 ⁵	8.1 ±1.6 x 10 ⁵	4.17±0.8 x 10 ⁵	0.1
% macrophages In BAL	12.2 ±3.3	6.6 ±2	10.1 ±2	N.S.
% neutrophils in BAL	88.6 ±1	91.2 ±2	87.3 ±5	N.S.
% lymphocytes in BAL	1.9 ±0.6	2 ±0.5	2.5 ±1	N.S.
Myeloperoxidase Activity/g lung tissue	18 ±4.7	* 36 ±4.5	29.6 ±3.1	0.018

Data are presented as mean ± SE. Statistical significance, indicated by p value in this table, was tested by one way ANOVA, followed by Tukey's post hoc test. n = 5 (differential leukocyte count), n=8 (all other parameters). * p < 0.05 statistically significant vs. casein (post-hoc test).

5.4.6. Conclusion of the preliminary study:

There is a tendency towards higher bacterial clearance in the pressurized whey-fed group relative to the native whey group without associated differences in the inflammatory response. Results of the analysis of MPO levels suggest a higher anti-inflammatory effect of casein than native whey. However MPO activity was not significantly different between the native and pressurized whey groups.

5.5. Principal Study:

Aim: This part of the study was undertaken to:

- 1) Finalize and complete the results of the preliminary study, which showed a trend towards decreased lung bacterial burden in the pressurized whey group. We added more experiments of native and pressurized whey fed groups only, infected them, and tested their pulmonary infection and inflammation levels at Day 3.
- 2) To explore the mechanisms behind this enhanced bacterial clearance in the pressurized whey group. These aspects included cytokine levels in BAL and serum, oxidation of BAL protein, total glutathione concentrations, elastase enzyme activity in BAL fluid, and bactericidal activity and superoxide anion production of neutrophils.

5.5. 1. Experimental design:

5.5.1.1. Day 3 experiment:

Four more supplementary experiments were conducted, in which mice were randomized exclusively to either native or pressurized whey-based diets, infected and

studied for the primary outcome measures of lung inflammation and infection at Day 3, as described earlier. Feeding and infection protocols used in the preliminary study were consistently followed. Each diet group was represented by 1-3 mice per experiment so that the four experiments were run in a paired manner, similarly to the 8 paired experiments run in the preliminary study. Primary outcomes (inflammatory response including BAL cell count and lung bacterial load) were determined and added to the corresponding results of the preliminary study so that the total number of observations, n=12, was analyzed. Supernatants of lavage samples from all the whey-based feeding experiments (native and pressurized whey, preliminary & complementary part of the study) were frozen in small aliquots at -80°C for later measurements of total glutathione, protein carbonyls, lipid per-oxidation, MIP-2 and GM-CSF.

5.5.1.2. Day 1 experiment:

Since lung bacterial burden was shown to be significantly lower in the pressurized whey group than in the native whey littermates, separate experiments were designed and undertaken to study the mechanistic aspects of neutrophil bacterial killing ability, in order to explain the observed enhanced innate defense in the pressurized whey group. These animals were studied only at Day 1 post-infection.

In those experiments (Day 1 post-infection), BAL inflammatory cells were collected and found to be 80-90% neutrophils, as determined by Diff-Quick staining. The inflammatory cells were then tested for their capacity to kill bacteria and to produce

superoxide anion as an index of respiratory burst strength. Lung bacterial burden and MPO activity were also assessed in lung tissue homogenates. Supernatants of BAL fluid were stored at -80°C for later measurements of protein carbonyls, lipid peroxidation and cytokine levels including granulocyte macrophage-colony stimulating factor (GM-CSF), interferon- γ (IFN- γ), interleukin- 1β , IL-4, IL-6, IL-10, IL-17, keratinocyte chemoattractant (KC) and macrophage inflammatory protein-2 (MIP-2), homologues to human interleukin-8 (IL-8), and tumor necrosis factor- α (TNF- α).

5.5.2. Measurements and assays:

5.5.2.1. MPO activity in lung tissue homogenates: MPO activity was measured in frozen samples of lung tissue using a kinetic method (for details see chapter 3 [3.3.4.1]).

5.5.2.2. Protein and lipid per-oxidation: ELISA-based method using Cell biolabs. Kit as described in chapter 4 (time course study).

5.5.2.3. Total glutathione in BAL fluid: colorimetric method and a standard curve of serial dilutions of glutathione, see details in chapter 4.

5.5.2.4. Elastase activity in BAL fluid:

As neutrophil elastase has been reported to kill *P. aeruginosa* through its catalytic activity (64), elastase activity in BAL supernatants of lavage fluid was measured according to Jayaraman and colleagues (176), with adaptations. Briefly, MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide (EPC No. FH237) substrate was dissolved in sulfoximide (0.04 ml, 25mM) and diluted 20-fold in Tris buffer. To 450 μL of this

diluted substrate, 50 µl of serial dilutions of elastase enzyme or BAL supernatant were added, mixed and left for 30 min in room temperature, and absorbance was read at 405 nm. Elastase activity was determined according to the readings of the standard curve and expressed as enzyme units/mg protein.

5.5.2.5. Bactericidal activity and superoxide anion production of neutrophils:

Method critique and adaptation: We aimed at harvesting neutrophils post-infection for the purpose of assessing bactericidal activity and superoxide anion production as early as possible. We were concerned that testing at later time points would not capture these anti-bacterial capabilities. We performed a time course of infection on a limited number of mice and collected BAL fluid at 5, 12, and 24 hrs post-infection (Figure 5.2) before performing these two assays on whey-fed animals. We did not find a high number of cells in BAL at 5 hrs; in addition, the differential staining showed a macrophage dominated response. The number of cells in BAL was high enough at 12 and 24 hrs ($0.5 - 1 \times 10^6$ cells/mouse BAL or more). Therefore, we chose the 24 hrs time point throughout subsequent experiments on native and pressurized whey pre-fed groups in order to ensure the highest number of cells possible in BAL (Figure 5.2).

5.5.2.5. A: The bactericidal activity of inflammatory cells (80-90 % neutrophils) was measured according to Hampton and co-workers (177, 178), with minor adaptations. Briefly, inflammatory cells (5×10^5 cells from each mouse lavage) were re-suspended in 0.5 ml. PBS containing 10% fetal bovine serum (FBS) and incubated with 5×10^7 CFUs of opsonized *P. aeruginosa* (suspended in 0.4 ml. of PBS containing 10% FBS). Aliquots of 50 µL were taken at 10 and 20 min,

diluted in autoclaved H₂O (pH 11), plated in triplicate on agar plates and incubated at 37°C overnight. Bacterial CFUs were counted and multiplied by

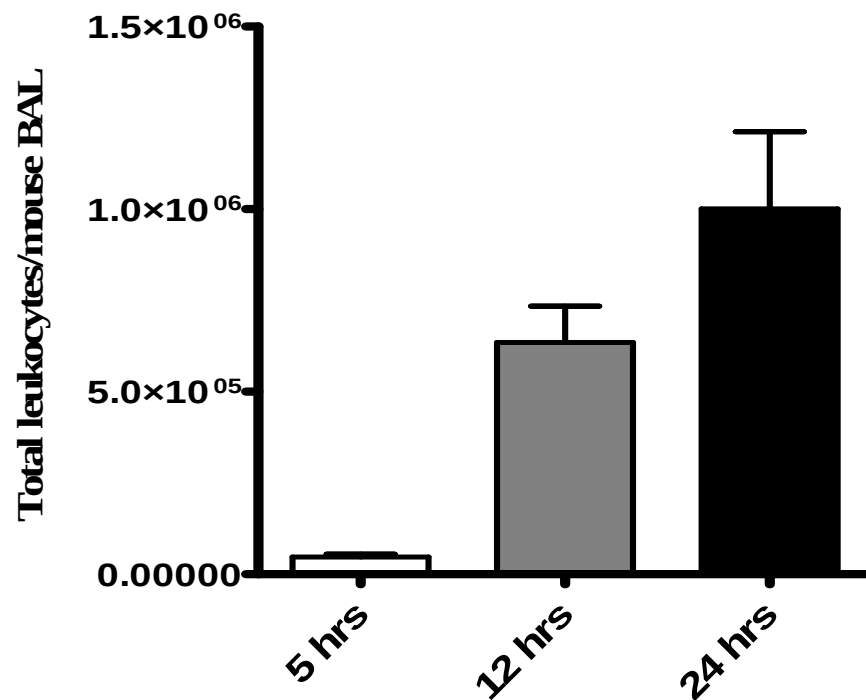


Figure 5.2: Time course of BAL inflammatory cells at 5, 12, and 24 hrs post-infection in chow-fed mice. At the beginning we tested this time course on 3 mice/group. More readings of BAL leukocyte counts were added to the 12 and 24 hr time points from the mice that were used to validate the bactericidal assay at 12 and 24 hrs. n=3 (5hrs), n=8-9 (12, 24 hrs).

the appropriate dilution factor. CFUs/ml At 10, 20 min. was calculated and normalized as % of the control tube (a tube containing opsonized bacteria and PBS instead of leukocytes), and plated at zero time, so that this sample represents a control and baseline (initial bacteria at time zero).

5.4.2.5. B: Superoxide anion production of neutrophils: Another way to assess bacterial killing ability of neutrophils is to evaluate the strength of their respiratory burst. Choosing this approach is of particular importance since the respiratory burst efficiency was shown by Jesaitis and co-workers (35), to be specifically important in neutrophil/biofilm bacteria interaction in this model. These authors concluded from their *in vitro* study on artificial biofilm that enhanced respiratory burst enables neutrophils to kill the bacteria before it escape from neutrophils, which are immobilized by the bacterial biofilm. The above referenced *in vitro* study supports the adaptation of the chosen method to the nature of *in vivo* pathogen-host interaction, at least theoretically.

Superoxide anion production was measured by inhibiting the reaction of ferricytochrome C by superoxide dismutase (SOD), as previously described (179). Briefly, 100 μ l HBSS (Hank's Balanced Salt Solution) and 50 μ l ferricytochrome C (2.7 mg/ml) were mixed in duplicate wells of a 96-well plate with or without 50 μ l SOD (1 mg/ml) as the baseline. In separate wells on the same plate a stimulus of 20 μ l of opsonized bacteria or 10 μ l PMA (10 μ g/ml) were added to HBSS and ferricytochrome C (with or without SOD). The plate was then incubated at 37°C for 10 min. A solution of 100 μ l of warmed PMNs suspension (10^6 /ml) were added to each well and incubated for 20 min at 37°C on a shaker. The microplate was then read

at 550 nm and the concentration of superoxide (nmol) was calculated using the following formula:

$$\text{nmol produced per 50,000 PMNs} = (\text{OD without SOD} - \text{OD with SOD}) \times 26.5$$

5.5.2.6. Cytokine assay:

Cytokines known to be implicated in the immune defense against pathogens (GM-CSF, IFN- γ , IL-1 β , IL-4, IL-6, IL-10, IL-17, KC, MIP-2 and TNF- α) were measured in supernatants of lung lavage samples at Day 1 post-infection using Mouse Fluorokine MAP Base Kit (R & D System) and Bio-Rad Bio-plex 200 system following the manufacturer's instructions and a standard curve for each cytokine. MIP-2 and GM-CSF were also assessed at Day 3. Detection ranges for these cytokines in Pg/ml were: GM-CSF: 8.8 - 6415, INF- γ : 22 - 6024, IL- 1 β : 61 - 43976, IL-4: 15.3 - 10640, IL-6: 10.4 - 7536, IL-10: 8.2 - 5804, IL-17: 20 - 14330, KC: 14 - 10359, MIP-2: 6.3 - 5076. Values that fell below the lower limit of the standard curve were given a value equal to the average of the lower limit in the standard curve and zero.

5.5.3. Statistical analysis

Results are presented as means $\pm$ SEM for normally distributed variables and otherwise presented as medians with inter-quartiles. One-way Anova test, followed by Tukey's post-hoc test were used to compare between the three diet groups in the preliminary study. Paired t-test or Wilcoxon matched paired test was used where appropriate to compare between the group means and medians at the same day post-

infection (Day 1 or Day 3) in the principal study (two groups only). Student-t test and Mann-Whitney test were used to analyze the statistical differences in superoxide anion production and bactericidal activity, respectively; they were also used, where appropriate, to compare between Day 1 and Day 3 post-infection in the same diet group. Statistical significance was considered when p was < 0.05 . Statistical analysis was conducted using Sigma Stat version 3.02.

5.5.4. Results

5.5.4.1. Clinical signs and Lung bacterial burden:

Weight loss, as a clinical sign in this model, was not different between the two diet groups at Day 1 or Day 3 post-infection (Table 5.3). Lung bacterial burden was not different between the groups at Day 1 post-infection; at Day 3, it was significantly lower in the pressurized whey group compared to the native whey counterpart ($p < 0.05$, Figure 5.3).

5.5.4.2. Lung inflammatory response:

Total inflammatory cell counts in BAL fluid were not significantly different between the two diet groups at Day 1 or Day 3 (Table 5.3). Percentage of neutrophils in BAL inflammatory cells was 80-90% in both groups at Day 1 or 3. Likewise, MPO activity in lung tissue, an indirect marker of neutrophil recruitment to the lung, did not differ between the diet groups at Day 1 or Day 3 (Table 5.3).

5.5.4.3. Protein carbonyls and lipid per-oxidation in BAL:

The level of protein carbonyls in BAL fluid supernatants was not significantly different between the diet groups at Day 1 post-infection (Figure 5.4). At Day 3 post-

infection, protein carbonyls were significantly lower in the pressurized whey group relative to the native whey group ($p < 0.05$, Figure 5.4). Further, they were significantly higher in the native whey group at Day 3 relative to Day 1 of the same diet group (native whey) ($p < 0.05$, Figure 5.4). In contrast, the protein carbonyls within the pressurized whey groups were not statistically different at Days 1 and 3 ($p > 0.1$, Figure 5.4). The levels of HNE-adducts were not significantly different between the diet groups either at Day 1 or Day 3 (data not shown).

5.5.4.4. Elastase activity in BAL fluid supernatants:

There was no significant difference in BAL elastase activity between the diet groups at Day 3 post-infection (means: 63.3 ± 6.6 vs. 70 ± 6.6 enzyme unit/mg protein in the native and pressurized whey groups, respectively, $n=11$, $p > 0.1$). Elastase activity in BAL correlated negatively with the lung bacterial burden in both native and pressurized whey groups; however, the negative correlation between BAL elastase and lung bacterial burden was greater in the pressurized whey group than the native whey group ($r = -0.89$ vs. $r = -0.58$, pressurized vs. native, $p = 0.08$, Table 5.4).

5.5.4.5. Total glutathione in BAL fluid supernatants: There was no statistically significant difference in BAL levels of total glutathione between the groups (means: 14.2 ± 3.8 and 14.5 ± 3.5 μM in the native and pressurized whey groups, respectively; $n=10$). However, BAL total glutathione concentrations tended to correlate with lung bacterial burden ($r=0.45$, $p=0.07$) in the pressurized whey group but not in the native whey group ($r=0.28$, $p=0.3$, Table 5.4).

Table 5.3: Inflammatory indices at Day 1, 3 post-infection in native and pressurized whey groups

Inflammatory index	Day 1		Day 3	
	Native whey	Pressurized whey	Native whey	Pressurized whey
Weight Change (% of base line)	- 6.55 ± 0.4%	- 6.88 ± 0.6%	- 16.5 ± 1.6 %	- 18.3 ± 1.2
BAL Total inflammatory cell count /ml	1.87 ± 0.34 x 10 ⁶	1.64 ± 0.18 x 10 ⁶	1.88 ± 0.4 x 10 ⁶	1.78 ± 0.2 x 10 ⁶
BAL % neutrophils	88.6 ± 2.6 %	82.2 ± 4.5 %	90.2 ± 1.3 %	86.2 ± 2.6
Myeloperoxidase Activity unit/g lung tissue	5 ± 2	6.5 ± 1.2	29.6 ± 4	28 ± 2.4

Data are represented as mean ± standard error. None of the above listed inflammatory markers showed a statistically significant difference between native and pressurized whey groups, either at Day 1 or Day 3. N=9 and 12 for Day 1 and Day 3, respectively (weight loss); 10 and 12 (inflammatory cells in BAL); 6 and 10 (% of neutrophils in BAL); 4 and 12 (MPO activity in lung tissue).

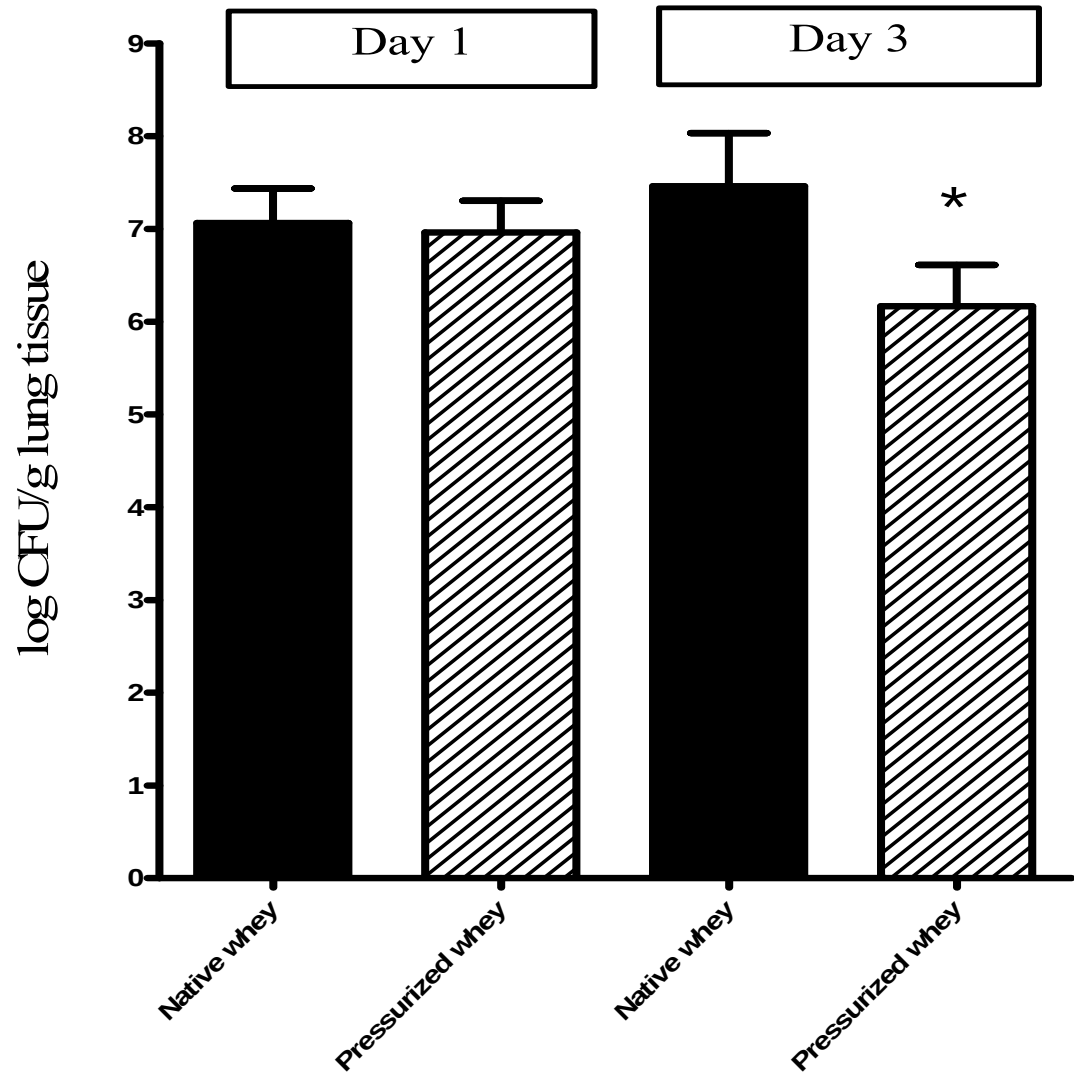


Figure 5.3: Lung bacterial burden presented as mean $\pm$ SE in native and pressurized pre-fed and infected mice. * Statistically significant difference between native and pressurized whey at Day 3, $p < 0.05$ paired t-test, $n=8$ (Day 1), $n=12$, (day 3), where n represents number of experiments, in which each diet group was represented by 1-3 mice per experiment.

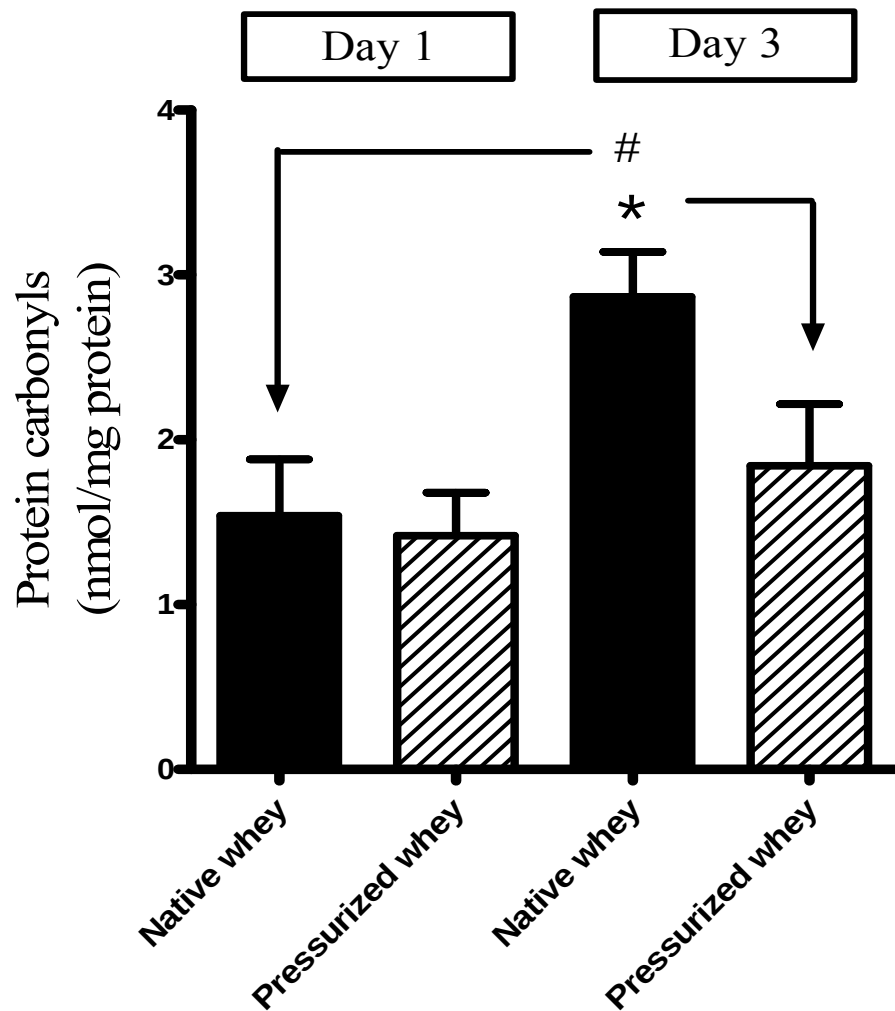


Figure 5.4: Protein carbonyls measured in the supernatants of lung lavage samples from native whey (solid bars) and pressurized whey (hatched bars)-fed mice at Day 1 or 3 post-infection. Data are presented as means $\pm$ SEM. * statistically significant difference between native and pressurized whey at Day 3 ($p < 0.05$), paired t-test. # statistically significant vs. native whey at Day 3 ($p < 0.05$), unpaired t-test. $n = 10$ (Day 1), $n=12$ (Day 3). Each point represents an independent experiment, in which each group was represented by 1-3 mice per experiment.

Table 5.4: Correlation between BAL elastase or total glutathione with lung bacterial burden

	Native whey			Pressurized whey		
Parameters, correlated at Day 3	r	p	n	r	p	n
BAL elastase activity/lung bacterial burden	- 0.58	0.03	13	- 0.89	p<0.001	16
BAL glutathione/lung bacterial burden	0.28	0.3	14	0.45	0.07	17

lung bacterial count. This negative correlation tended to be stronger in the pressurized whey group (p=0.08). Glutathione levels in BAL tended to correlate with lung bacterial burden in the pressurized whey group.

5.5.4.6. Bactericidal activity and superoxide anion production:

Bactericidal activity and superoxide anion production were measured at Day 1 post-infection to investigate early defense mechanisms that led to the lower bacterial burden in the pressurized whey group at Day 3 post-infection compared to the native whey group.

5.5.4.5. A. Bactericidal activity: There was a significant increase in leukocyte killing ability observed in the pressurized whey group (Figure 5.5).

5.5.4.5. B. Superoxide anion production: There was a significant increase in superoxide anion production in the pressurized whey-fed group compared to their native whey-fed counterparts in response to bacteria (Figure 5.6). Further, the production of superoxide anion tended to show a negative correlation with bacterial burden in the pressurized whey group ($r = -0.8$, $p = 0.08$, $n = 5$) but not in the native whey group ($r = -0.25$, $p > 0.1$, $n = 4$). There were no significant group differences in superoxide anion production when cells were stimulated with buffer or PMA (data not shown).

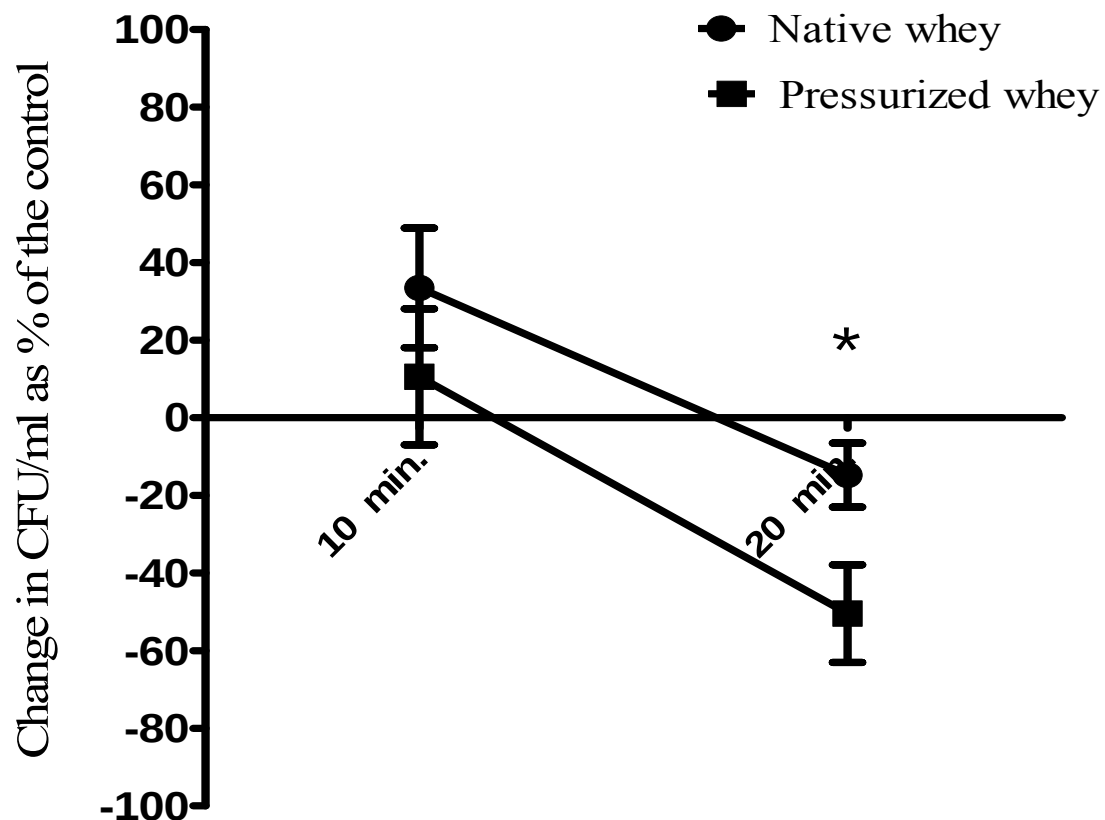


Figure 5.5: Estimation of bactericidal activity of inflammatory cells (80-90% neutrophils), at 24 hrs post-infection, from *P. aeruginosa*-infected mice, pre-fed with native or pressurized whey-based diets. 5×10^5 cells of BAL fluid were incubated with 5×10^7 CFUs of opsonized bacteria and the number of surviving bacteria were estimated at 10 and 20 minutes post-incubation. Points represent means $\pm$ SEM. A significant decrease in the percentage of the surviving bacteria was evident at 20 minutes in the pressurized whey group (squares) compared to the native whey group (circles). * $p < 0.05$, t-test, $n=9-12$.

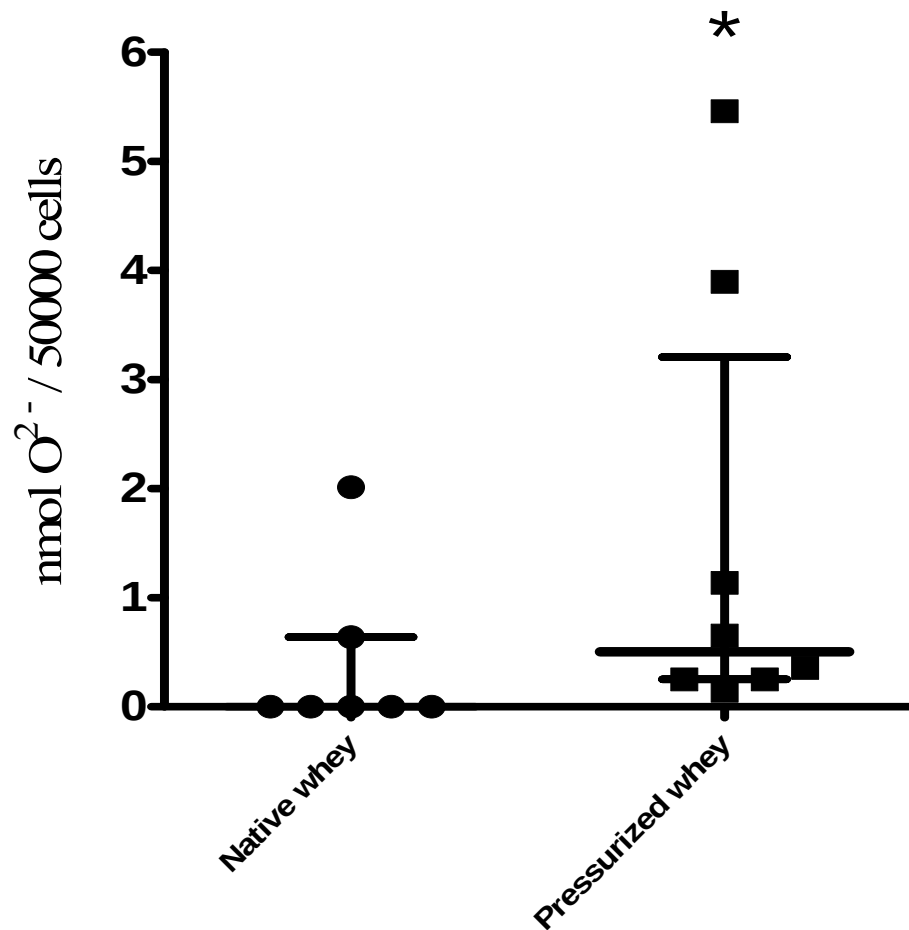


Figure 5.6: Leukocytes (80-90% neutrophils) from lung lavage were tested for their level of superoxide anion production as an index of respiratory burst strength. * Statistically significant higher levels of superoxide anion were observed in leukocytes from the pressurized whey group (squares) compared to the native whey group (circles). ($p < 0.05$, $n=7-8$). Data are presented as median and inter-quartiles.

5.5.4.7. Cytokines in BAL supernatants and serum:

BAL levels of GM-CSF, IFN- γ , IL-4, IL-6, IL-10, IL-17, KC and TNF- α were not different between native and pressurized whey groups at Day 1 post-infection (data not shown, except for GM-CSF, shown in Figure 5.9). IL-1 β ($p=0.09$) (Figure 5.7) and MIP-2 ($p=0.06$) (Figure 5.8) tended to be lower in the pressurized whey group at Day 1. Further, MIP-2 also tended to be lower in the pressurized whey group at Day 3 ($p=0.1$) (Figure 5.8). Day 3 GM-CSF concentrations were 10-fold lower in the pressurized whey group ($p < 0.05$) (Figure 5.9). Moreover, GM-CSF levels were decreased significantly at Day 3 versus Day 1 in the pressurized whey group ($p < 0.001$), whereas no statistically significant decrease was observed in GM-CSF levels between Day 3 and Day 1 in the native whey group ($p > 0.1$). Levels of IL-6, MIP-2 and KC were tending to be less in the sera of the pressurized whey group ($p=0.1$, Figures 5.10, 5.11).

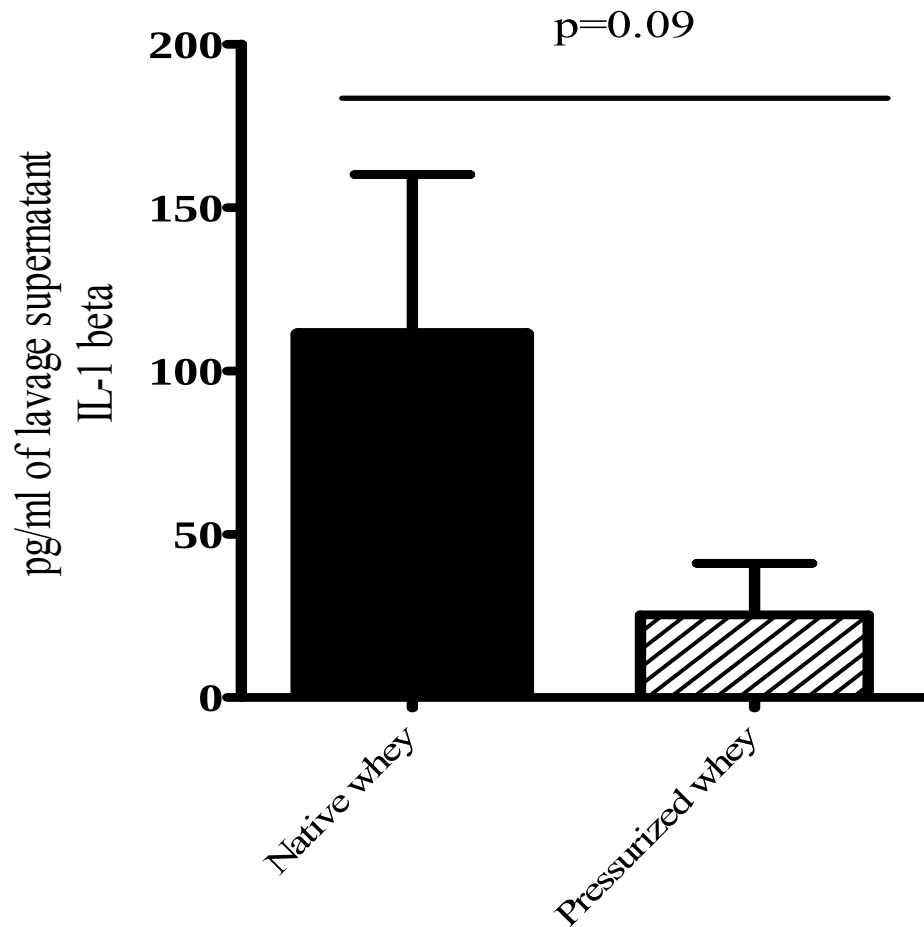


Figure 5.7: Levels of IL-1 β in lung lavage fluid supernatant from the native and pressurized whey groups at Day 1 post-infection. IL-1 β tended to be less in the pressurized whey group compared to their native whey-fed littermates ($p=0.09$). Data are pooled from 7 different experiments that were run under the same conditions ($n=7$).

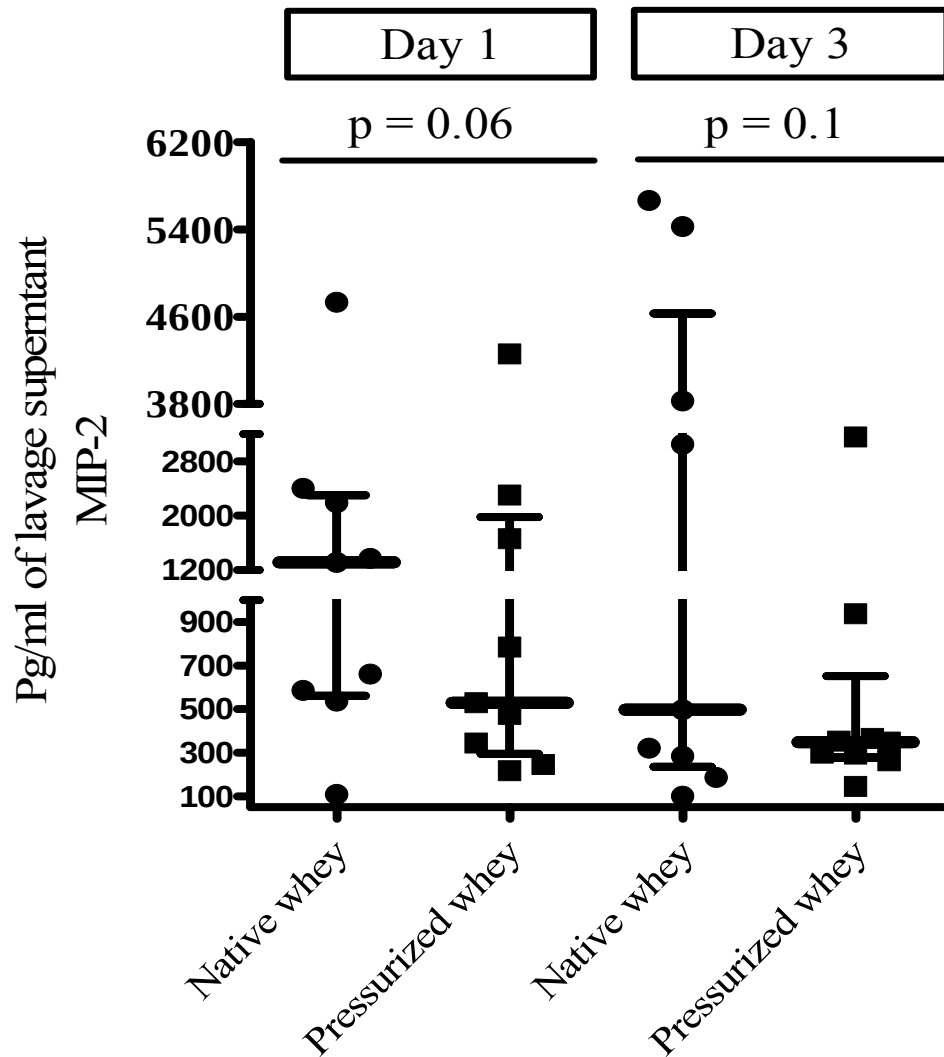


Figure 5.8: Levels of MIP-2 in BAL of native and pressurized whey-fed groups at Day 1 and Day 3 post-infection. Comparing levels of MIP-2 in the native (circles) and pressurized whey (squares) groups within Day 1 or Day 3 showed a tendency towards lower MIP-2 concentrations in the pressurized whey group relative to the native whey group at both days of infection (Day 1: $p=0.06$; Day 3: $p=0.1$). Data are expressed as medians with inter-quartiles ($n=9$ for all groups). Each point represents an independent experiment, in which each group was represented by 1-3 mice. Data are presented as median and inter-quartiles.

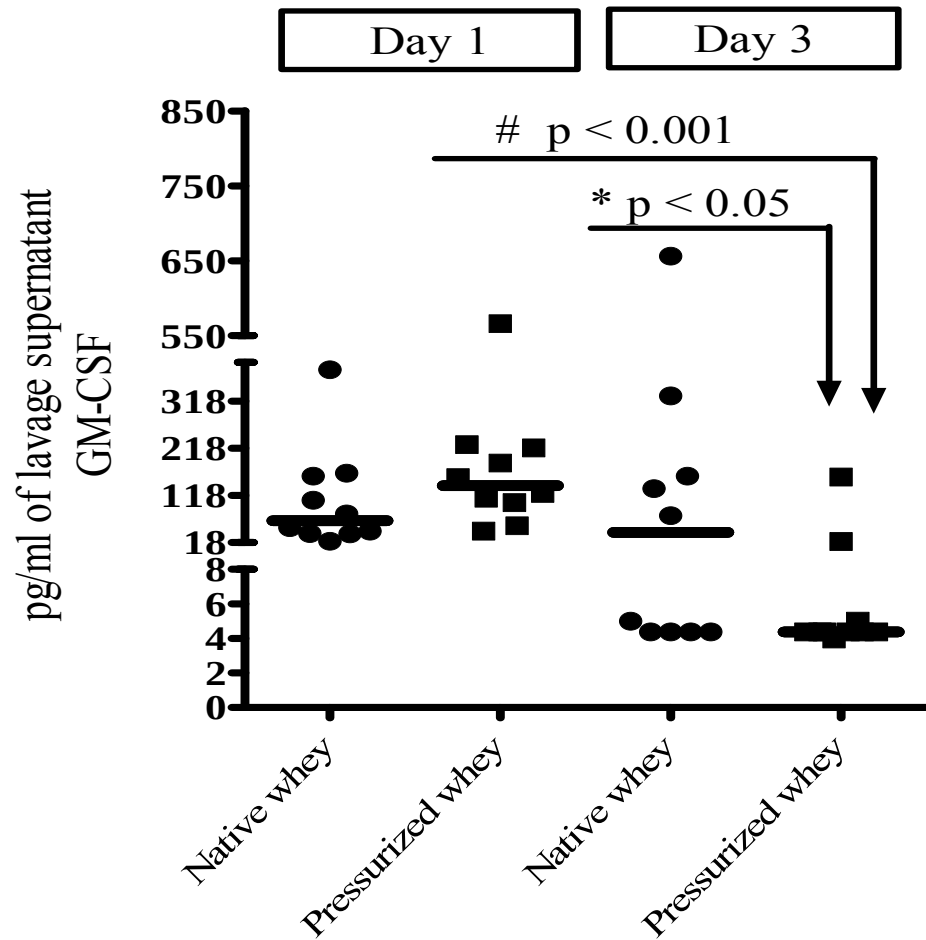
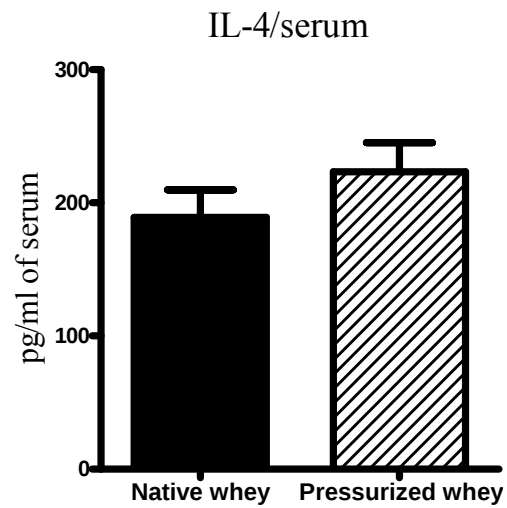


Figure 5.9: GM-CSF was measured in the supernatant of lung lavage samples from the native whey protein group (circles) and the pressurized whey protein group (squares) at Day 1 and Day 3 post-infection. Data are expressed as medians with inter-quartiles. GM-CSF at Day 3 post-infection was significantly lower in the pressurized whey group compared to the native whey group (* $p < 0.05$) and compared to Day 1 within the same diet group (# $p < 0.001$). The native whey group showed a non-significant decrease between Day 1 and Day 3 ($p > 0.1$) ($n=10$ for all the groups). Each point represents an independent experiment, in which each group was represented by 1-3 mice.

5.10. A.



5.10. B.

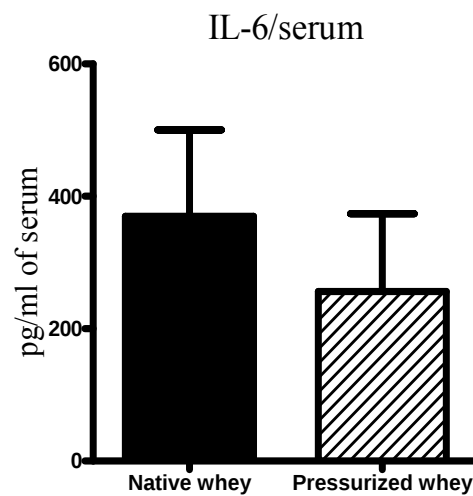
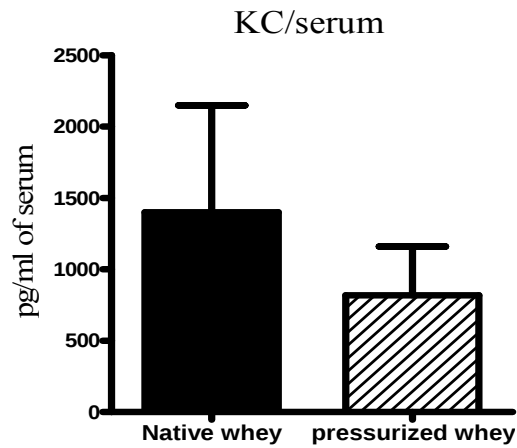


Figure 5.10: Cytokine levels in sera of native and pressurized whey pre-fed groups at 24 hrs post-infection. Unlike IL-4 (A), IL-6 (B) tended to be lower in the pressurized whey group ($p=0.1$, paired t-test, $n=4$).

5.11. A



5.11. B.

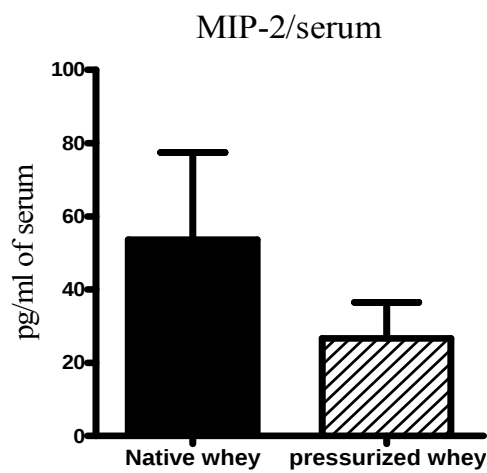


Figure 5.11: Cytokine levels in sera of native and pressurized whey pre-fed groups at 24 hrs post-infection. There is a trend towards lower KC (A) and MIP-2 (B) levels in sera of the pressurized whey group ($p=0.1$, paired t-test, $n=4$).

5.5. 5. Discussion

The present findings demonstrate that mice fed pressurized whey protein exhibited more efficient bacterial clearance than mice fed native whey protein. This was not associated with higher total or differential inflammatory cells in bronchoalveolar lavage fluid, suggesting that enhanced bacterial clearance in mice pre-fed with pressurized whey, is not mediated through higher transmigration of neutrophils to the site of infection (lungs), but through more efficient bacterial clearance (180). Enhanced bacterial clearance was associated with an increased ability to kill bacteria and a greater superoxide anion response to bacteria in the pressurized whey fed group. Pressurized whey supplementation also reduced the BAL protein oxidation typically induced by *P. aeruginosa* infection (8-10).

In Cystic Fibrosis, there is a need for enhanced bacterial clearance without overly exuberant oxidation. An enhanced intracellular superoxide burst induced by pressurized whey protein supplementation may increase the efficiency of neutrophil killing ability and limit lung damage caused by chronic *P. aeruginosa* infection. Supplementation with pressurized WPI may thus help reverse the reduced oxidative burst that occurs in neutrophils exposed to *P. aeruginosa* in biofilms, as occurs in Cystic Fibrosis (181). This oxidative burst can enhance bacterial killing by neutrophils trapped in the biofilm (35).

It is interesting to note that the superoxide burst was enhanced when neutrophils from pressurized whey protein-fed mice were exposed to bacteria, but not to PMA. This suggests that pressurized whey may enhance the response to bacterial exposure

through pathways, such as by acting on cell surface receptors, that work in conjunction with protein kinase C for the oxidative burst (182).

Pretreatment of human neutrophils with whey protein extract, as well as with individual whey protein components such as β -lactoglobulin or α -lactalbumin, was found to increase their superoxide anion production in response to fMLP (182). Milk protein-derived peptides were also shown in an *in vitro* study to increase superoxide anion production of neutrophils through ligation with their receptors (132). Tripeptides such as GLF generated from residues 51-53 of α -lactalbumin have been shown to stimulate superoxide anion production by human neutrophils (132). Miglipore-Samour and colleagues (132) have suggested that different types of peptide spectra will stimulate the oxidative burst to different degrees. In this regard, it is possible that the changes in peptide profiles generated from the digestion of pressurized whey protein (19) can result in immunomodulatory effects such as enhanced oxidative burst and greater bacterial killing ability. The production of bioavailable antibacterial peptides from the digestion of pressurized whey could also contribute to enhanced bacterial clearance (19). Of note that post-supplementation body weight gain as well as food consumption by mice was not different between the two diet groups, suggesting that the protective effect of pressurized whey protein may not be explained by, or related to, differential nutritional values. However this observation and interpretation might not necessarily apply to CF patients since we know that our model bypasses the digestive difficulties and the related nutritional challenges seen in CF.

Another contributing factor to the enhanced bacterial clearance in the pressurized whey group may be the high concentration of glycomacropeptides in the

Inpro90 WPI formulation used in the present study in combination with the enhanced pepsin digestion of pressurized whey proteins (19). Li and Mine (18) have shown that the phagocytic activity of the macrophage cell line U937 was increased by pepsin-digested glycomacropeptides relative to the non-digested glycomacropeptides.

The greater inverse correlation of elastase and bacterial burden observed in the pressurized whey protein supplemented group suggests a more efficient and effective killing of bacteria by elastase, known to be lethal for *P. aeruginosa* (64).

While BAL glutathione concentrations did not differ between the diet groups, the trend towards a positive correlation with bacterial burden in the pressurized whey protein-fed mice suggests that pressurized whey protein intake could support an induced glutathione response to *P. aeruginosa* lung infection previously noted by Day and co-workers (157). This may have contributed to limiting BAL protein oxidation that would help preserve anti-elastase activity, which is defective in Cystic Fibrosis (183).

IL-1 β , an inflammatory cytokine, tended to be less in the pressurized whey group relative to the native whey group. Interestingly, absence or blockade of the IL-1 β receptor in a murine model of *P. aeruginosa* infection reduced bacterial burden (184). This suggests that the mild reduction in lavage IL-1 β in the pressurized whey group may have contributed to the observed enhanced bacterial clearance.

IL-8 is elevated in CF airways (185, 186), and in a similar fashion, BAL MIP-2 (a mouse homologue of IL-8) is increased in a murine model of *P. aeruginosa* lung infection, similar to the model used in this study (157). IL-8 is the primary chemoattractant of neutrophils to the lungs. The trend towards lower levels of MIP-2 is

indicative of an anti-inflammatory effect of supplementation with pressurized whey. In this regard, a one-month pilot study of pressurized whey supplementation in children and adults with CF demonstrated a trend towards a decrease in whole blood stimulated IL-8 responses (115).

We observed a decrease in GM-CSF concentrations in BAL supernatants at Day 3 in the pressurized whey fed group. GM-CSF stimulates the clonal growth of neutrophils and activates them in the vicinity of infection (187). It is secreted by the airway epithelial cells in response to mucoid strains of *P. aeruginosa*, and enhances the longevity of neutrophils (188). GM-CSF is elevated in CFTR knockout mice infected with *P. aeruginosa* in comparison to infected wild type mice at Day 3 (143). CF patients also demonstrate elevated GM-CSF concentrations in their lung secretions (189). The observed decrease in GM-CSF with pressurized whey supplementation could be beneficial for patients with Cystic Fibrosis.

In conclusion, the present study demonstrates that pressurized whey protein supplementation enhances bacterial clearance and neutrophil activity, while limiting airway protein oxidation. These effects may help limit the severity of lung infection.

CHAPTER 6

Supplementation with Pressurized Whey Protein Increases Systemic Neutrophils in Response to Pulmonary Infection in Mice

6.1. Background and rationale:

Neutrophils play an important role in combating bacterial infection; therefore patients who develop neutropenia following chemotherapy or bone marrow transplantation are more vulnerable to bacterial infection, with a higher risk of mortality compared to subjects with normal neutrophil counts (190, 191). Moreover, low absolute neutrophil counts have been used as a marker to predict the risk of sepsis in new born infants (192). Blood neutrophil counts rise in infected patients compared to healthy controls (193). Blood depleted of neutrophils *ex vivo* has an impaired ability to resist the growth of *Mycobacterium bovis* and *Mycobacterium tuberculosis* by 7.3- and 3.1-fold, respectively (194). All together, an elevated absolute neutrophil count is evidently an important line of defense against invading bacterial pathogens. Peptides found in whey protein components, such as GLF (an α -lactalbumin-derived tripeptide) enhanced the proliferation of a macrophage-like cell line *in vitro*. Likewise, glycomacropeptide (GMP, a whey component found at a high concentration in the whey protein formulation used in this study) stimulated the proliferation of a macrophage-like cell line *in vitro* (18), induced neutrophilia, and decreased spleen

bacterial load in infected mice *in vivo* (99). Pepsin-digestion of GMP augmented its ability to stimulate the proliferation of a macrophage cell line *in vitro* to a greater extent, compared to the undigested GMP (18). Pressurization of whey proteins enhances their pepsin-digestibility; therefore we hypothesized that pressurization will augment their stimulatory effect on leukocyte proliferation. This is expected to render the pressurized whey formula superior to its native whey counterpart in enhancing the innate immune defense against microbes.

6.2. Materials & Methods:

Mice were fed native or pressurized whey protein-based diets for 4 weeks, infected with 8×10^5 CFUs/mouse of *P. aeruginosa* and their total and differential leukocyte blood count was estimated at Day 1 post-infection. Blood was collected by cardiac puncture, and a sample of blood about 120 μ l was transferred to a small vial containing heparin as an anti-coagulant. 100 μ l of this blood sample was used for total blood leukocyte count using a Vet ABC machine at the diagnostic laboratory, McIntyre animal facility, McGill University. Blood smears were prepared from the collected blood samples, dried, and stained using Wright's stain for differential count. A total of 100 leukocytes were differentially scored per sample by a technician who was blinded to the study. Absolute count was extrapolated by multiplying the percentage of the differential count by the total leukocyte count.

6.3. Results and data analysis:

The absolute number of neutrophils was compared between native and pressurized whey pre-fed groups. The absolute neutrophil count in the blood of mice in each diet group was then compared separately to its corresponding values in a chow-fed, non-infected group as a baseline control representing the non-challenged healthy mice.

The absolute neutrophil count in the blood at Day 1 post-infection tended to be higher in the pressurized whey-fed group compared to the native whey-fed counterpart ($p=0.1$). Further, comparison of the absolute count of blood neutrophils in the pressurized whey group with the correspondent count of the chow non-infected control group revealed a significant rise in absolute blood neutrophils in the pressurized whey group (Figure 6.1). In contrast, the absolute neutrophil count of the infected native whey group was not significantly different from the non-infected chow (Figure 6.1).

Table 6.1: Total and differential blood leukocyte count in native, pressurized whey and chow-fed non-infected groups (Day 1 post-infection).

	Total leukocyte count in blood $\times 10^6/\text{L}$	% Neutrophils	% Lymphocytes	Absolute neutrophil count $\times 10^6/\text{L}$
Native whey 24 hrs post- infection	3.88 $\pm$ 0.6	18.5 $\pm$ 3.4	80.8 $\pm$ 3	0.62 $\pm$ 0.1
Pressurized whey 24 hrs post- infection	4.37 $\pm$ 0.6	24.2 $\pm$ 5.4	75.5 $\pm$ 5	1.04 $\pm$ 0.2
Chow non-infected	9 $\pm$ 0.8	3.75 $\pm$ 1	96.2 $\pm$ 1	0.35 $\pm$ 0.1

Data are expressed in means $\pm$ SE.

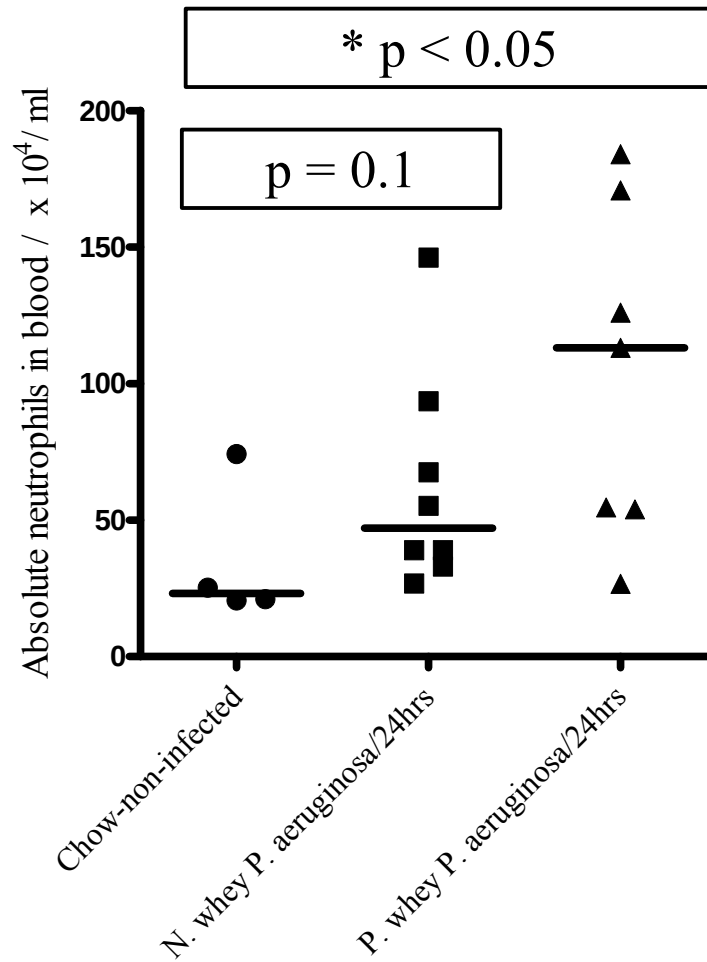


Figure 6.1: Absolute neutrophils tended to be higher in the pressurized whey group ($p=0.1$). Comparing the absolute neutrophils in the native or pressurized whey groups separately to the absolute neutrophil count of the non-infected chow-fed group showed a statistically significant rise in blood neutrophils in the pressurized whey group vs. the non-infected group ($p< 0.05$), while the absolute neutrophil count of the native whey group was non-significantly different from the non-infected group ($n=4$ for non-infected mice, $n=7-8$ for infected native or pressurized whey groups).

6.4. Discussion and conclusion:

Neutrophils are known to be responsible for the innate immune defense; as such, they migrate from blood to the site of infection in order to kill microbes. We observed an increased percentage as well as absolute neutrophil numbers at the expense of lymphocyte percentage and absolute numbers in the blood of infected mice compared to uninfected mice. Our data shows that both total leukocyte count and the percentage of neutrophils in blood tended to be higher in the pressurized whey group at Day 1 post-infection compared to their native whey littermates. GMP, a component of whey, increased blood neutrophils in a mouse model of sepsis. Feeding with an α -whey protein-based diet (α -whey contains all but β -LG components of unfractionated whey) increased total blood leukocytes and neutrophils in *Eimeria vermiciformis*-infected mice compared to casein- and soy bean-fed littermates (172). The beneficial role of an increase in blood neutrophils was reported in a mouse study with pulmonary infection with *P. aeruginosa*-loaded beads by Jensen and co-workers (2004) (180). These authors studied neutrophil activation in a similar lung infection model in susceptible (BALB/c) and resistant C3H/HeN mice, and found that the resistant mice exhibited faster neutrophil activation and more efficient bacterial clearance at Day 2 post-infection, and had a higher percentage of neutrophils in their blood (180), compared to the susceptible strain, which showed a slower bacterial clearance.

In conclusion, this chapter indicates, at least at the preliminary level, that supplementation with pressurized whey proteins can enhance the neutrophil reservoir in the blood as a response to infection.

CHAPTER 7

Conclusion, Limitations and Future directions

7.1. Conclusion:

This thesis work examined and validated the time course of infection, inflammation and protein oxidation in a murine chronic infection model with *P. aeruginosa*. We found that inflammation and infection started to diminish after Day 3 post-infection, and waned by Day 7, while protein oxidation in the airways was evident at all time points. These results mimic to some extent what happens in CF patients where the inflammation persists even after clearance of the infection.

One of the main findings of this study is the enhanced bacterial clearance as indicated by the lower lung bacterial burden at Day 3 post-infection in the pressurized whey-fed group compared to their native whey-fed counterparts, without a concomitant attenuation in the inflammatory indices (e.g. weight loss, inflammatory cells in BAL etc.). This supports that the inflammatory response is more efficient in the pressurized whey protein-fed group than its native whey counterpart. To this end, we decided to test some of the mechanisms that would reflect the efficiency of neutrophils to kill bacteria in both diet groups. As expected, we found enhanced bactericidal activity as well as superoxide anion production (an indicator of the strength of the respiratory burst) by neutrophils from the pressurized whey-fed group compared to their native whey counterparts.

Although the inflammatory indices at the primary outcome level (e.g. weight loss, inflammatory cells in BAL etc.) were not different between the two diet groups at Days 1 or 3 post-infection, we observed a significant decrease in the inflammatory cytokine GM-CSF at Day 3 and a tendency towards lower IL-1 β (at Day 1), lower MIP-2 (at Days 1 and 3) in the airways of the pressurized whey-fed mice compared to their native whey counterparts. Given that these cytokines are known pro-inflammatory mediators, their decreased levels in the pressurized whey group are beneficial to the host since these decreases were not associated with compromised bactericidal activity. In fact, the bactericidal activity of neutrophils from the pressurized whey group was even higher than that of the native whey group.

We also observed less protein oxidation at Day 3 in the pressurized whey group compared to the native whey counterpart. This suggests that pressurized whey may help to improve bacterial clearance by protecting proteins biologically implicated in this process, from oxidative damage.

Finally, we observed an increased neutrophil count in the blood of pressurized whey-fed mice at Day 1 post-infection, compared to their native whey-fed counterparts. A higher systemic neutrophil count could be beneficial to the host during an infectious challenge at an early time point (Day 1 post-infection), as neutrophils are responsible for the early innate defense mechanisms.

In conclusion, the present thesis demonstrates through a series of ideas, experiments and analyses that supplementation with pressurized whey protein enhances innate immune defense to a greater extent than does its native counterpart.

7.2. Limitations

1. Although this model of infection does not recapitulate all the typical clinical features and phenotypes of CF patients, it is still relevant in representing certain immune responses that occur in prolonged pulmonary infection in CF and COPD as discussed in Chapter 3.

2. The host immune response, studied in this model of *P. aeruginosa*-impregnated beads represents the host defense and inflammatory response to an established prolonged infection where the agar (matrix of beads) seems to slow the growth and immediate release of bacteria in the early time window (e.g. 2 or 4 hrs post-infection). That is why we did not see a massive neutrophil recruitment at 5 hrs post-infection (Chapter 5). As such, this model does not mimic the early immune response (e.g. early pathogen/epithelium interaction) at the early time window of 2-4 hours as seen when using an acute infection model with planktonic bacteria (195).

3. Due to the limited cell collected from a single mouse BAL ($0.5 - 1 \times 10^6$), it was not possible to continue the approach to the 2nd phase of phagocytosis on the few cells remaining after bactericidal activity. Unlike the original method where the authors used 10 ml. of blood from patient to collect neutrophils, an average of 1 ml. of mouse blood from a single mouse was not sufficient to adapt this method from blood neutrophils. Likewise these limited cell numbers from a mouse BAL were used for measurement of superoxide anion only. While some authors pool cells from several mice, the variability in response may be lost. Given that there is variation in infectious dose inoculums, we felt that it was more important to compare within day responses.

4. A standard chow diet contains 18.1% protein, comparable to the 20% protein in the whey-based semi-purified diets and has been used as a non-whey control in other feeding studies (101, 102, 173). However, chow contains less fat than the semi-purified whey-based diets (fat, 14 % in whey-based diet vs. 4.5 % in chow). The higher fat content in the semi-purified diets was done expressly to more closely mimic human diets. This higher fat content may alter the inflammatory responses. For instance, this may give rise to lipid peroxidation, and worsen the degree of oxidative damage.

7.3. Future Directions

The overall objective of this thesis was to test the hypothesis that pressurized whey enhances innate immune defense more efficiently, compared to the native whey. Although the results and conclusion of the thesis support the above-mentioned hypothesis, further mechanistic studies addressing the above mentioned hypothesis, as well as clinical trials, would be legitimate and justified. Among the suggested mechanistic aspects:

The overall objective of this thesis was to test the hypothesis that pressurized whey enhances innate immune defense more efficiently, compared to the native whey. Although the results and conclusion of the thesis support the above-mentioned hypothesis, further mechanistic studies addressing the above mentioned hypothesis, as well as clinical trials, would be legitimate and justified. Among the suggested mechanistic aspects:

1. Studying infection and the inflammatory response at a time point between Days 3 and 7 (e.g. day 4 or 5): Since the time course study of this thesis showed that

infection and inflammation are strong at Day 3 and that they are attenuated at Day 7, studying a time point such as Day 4 or 5 may unravel anti-inflammatory effects of pressurized whey protein at the level of the primary outcomes (such as earlier recovery from the peak of body weight loss at Day 3 and/or detection of lower BAL inflammatory cells and neutrophils in the pressurized whey group) that were not seen at Day 3 in the present study.

2. Since the present study showed that the inflammatory cytokines IL-1 β and MIP-2 tended to be lower in the airways of the pressurized whey-fed group at Day 1; and that IL-6, KC and MIP-2 tended to be lower in serum of the pressurized whey group at Day 1, the measurement of these inflammatory cytokines in a larger number of samples from mice pre-fed native or pressurized whey would further confirm the decreased concentrations in these inflammatory cytokines. If the decrease in the levels of these cytokines is confirmed (lower MIP-2, IL-1 β in BAL; lower IL-6, KC and MIP-2 in serum), then testing the gene expression of these cytokines (by measuring mRNA, messenger ribonucleic acid) in the cellular pellet of BAL fluid as well as lung tissue should follow. If mRNA measurements show a decreased expression parallel to the decreased concentrations of these cytokines in BAL, then evaluating the activation of the transcription factors NF- κ B and AP-1 in the cellular pellets of BAL would be the next valid mechanistic aspect to be investigated. Since pressurized whey has enhanced anti-oxidant potential (19, 128) and given that oxidative stress affect inflammatory cytokines through the above mentioned transcription factors, then investigating them would be reasonable (196-198).

3. The present study did not demonstrate a difference in the total number of BAL inflammatory cells or in the percentage of neutrophils in BAL cells between native and pressurized whey groups. However, bacterial clearance was enhanced in the pressurized whey group; this indicates a more efficient bacterial clearance in the pressurized whey group that does not implicate faster or increased migration of neutrophils to the lung or a higher expression of adhesion molecules (180); but likely indicates a possible faster activation of neutrophils from the pressurized whey group relative to the native whey group. It is therefore legitimate to examine the expression of CD11b and CD62, the activation markers of neutrophils, at early time points such as Days 1 and 2 as reported previously in a similar beads model (180).
4. Since the present study showed higher bactericidal activity in the pressurized whey protein-fed group, examining the phagocytic and killing ability of neutrophils (the two components of bactericidal activity) separately would further dissect the higher efficiency of bacterial clearance in the group pre-fed with pressurized whey. Although increased superoxide production relates to killing ability, yet testing phagocytosis in native and pressurized whey-fed mice is an additional valid approach.
5. Enhanced superoxide production by pressurized whey supplementation may be further verified at the mechanistic level by assessing NADPH oxidase activity in neutrophils from pressurized vs. native fed-mice *ex vivo* as described by Rusu et al. 2009 (14).

6. Since the pressurized whey intervention was introduced through the gastrointestinal tract to be absorbed and reach the systemic circulation, and not presented locally to the lungs, one can assume that the enhanced phagocytosis and killing ability of neutrophils can be reflected and examined systemically in blood neutrophils or neutrophils from peritoneal lavage, by flow cytometry, as previously reported. This would broaden the implications for pressurized whey to include other disease models such as sepsis.
7. Based on the increased protein oxidation (protein carbonyls) at Day 3 in the native whey group, and considering the antioxidant potential of pressurized whey protein, it would be worthwhile to identify specific oxidized proteins such as oxidized surfactant protein-D (SP-D) , known to be implicated in bacterial clearance. Increased oxidants are also expected to induce apoptosis of neutrophils, therefore it is legitimate to assess neutrophil apoptosis in BAL fluid at Day 1 and/or 3, using appropriate immunological & histological approaches on BAL cytopsin slides.
8. Diaphragm contractility is significantly and preferentially compromised in prolonged pulmonary infection with *P. aeruginosa* (138), due to the inflammatory response. It would be reasonable to study the effect of pressurized whey supplementation on diaphragm contractility and protein oxidation in this model.
9. The validation of the beneficial anti-microbial effect of pressurized whey protein in another animal infection model such as sepsis and acute infection (pneumonia) models would further confirm our findings, and show a broader protective effect of pressurized whey protein. In this regard, a sepsis model would be a suitable one.

10. Given that supplementation with pressurized whey protein improved lung function and nutritional status in children with CF (115), improved lung function and quality of life in COPD patients (131) and given that supplementation with pressurized whey protein limited bacterial burden in a murine model of *P. aeruginosa* lung infection, mimicking infection in CF patients, in the present study, it would be interesting to test whether supplementation with pressurized whey reduced *Pseudomonas aeruginosa* burden in CF patients.
11. If the blood neutrophil count at Day 1 post-infection is further confirmed in the agar bead model of pulmonary infection then measurement of granulocyte-colony stimulating factor (G-CSF), a cytokine known to stimulate proliferation of granulocytes precursors (199), mediate neutrophil release from bone marrow in this model of lung infection (200), would be reasonable. Measurement of this cytokine in serum at early time points such as Day 1 and/or 2 post-infection will answer the question as to whether there is quantitatively more synthesis of this cytokine in the sera of pressurized whey pre-fed animals. If this is demonstrated, then femoral bone marrow neutrophil counts (in addition to peripheral blood counts) will help to indicate whether G-CSF works through enhanced bone marrow release or enhanced bone marrow production (200).

7.4. Clinical and therapeutic implications:

The mucoid strain of bacteria can mutate to develop antibiotic resistance, which is associated with a poor prognosis. Pressurized whey can enhance the innate immune defense through mechanisms that differ from antibiotics. Moreover, pressurized whey protein is expected to improve the nutritional status

and general well being of CF and COPD patients as reported (115, 131). Furthermore, no serious or prevailing side effects have been reported from pressurized whey consumption. This makes it a feasible promising potential therapeutic approach. Further validation of the anti-microbial properties of pressurized whey protein could lead to potential applications as a nutritional supplement for:

1. Infected CF patients, to help control chronic infection, and non-infected patients, to mitigate their persistent inflammation and promote resistance to infection and avoid the subsequent conversion of an acquired acute infection to a chronic one.
2. COPD and bronchiectasis patients, to enhance their resistance to infection and attenuate the oxidative stress known to be associated with COPD.
3. HIV patients, since their immune function is compromised.
4. Subjects or patients vulnerable to sepsis.

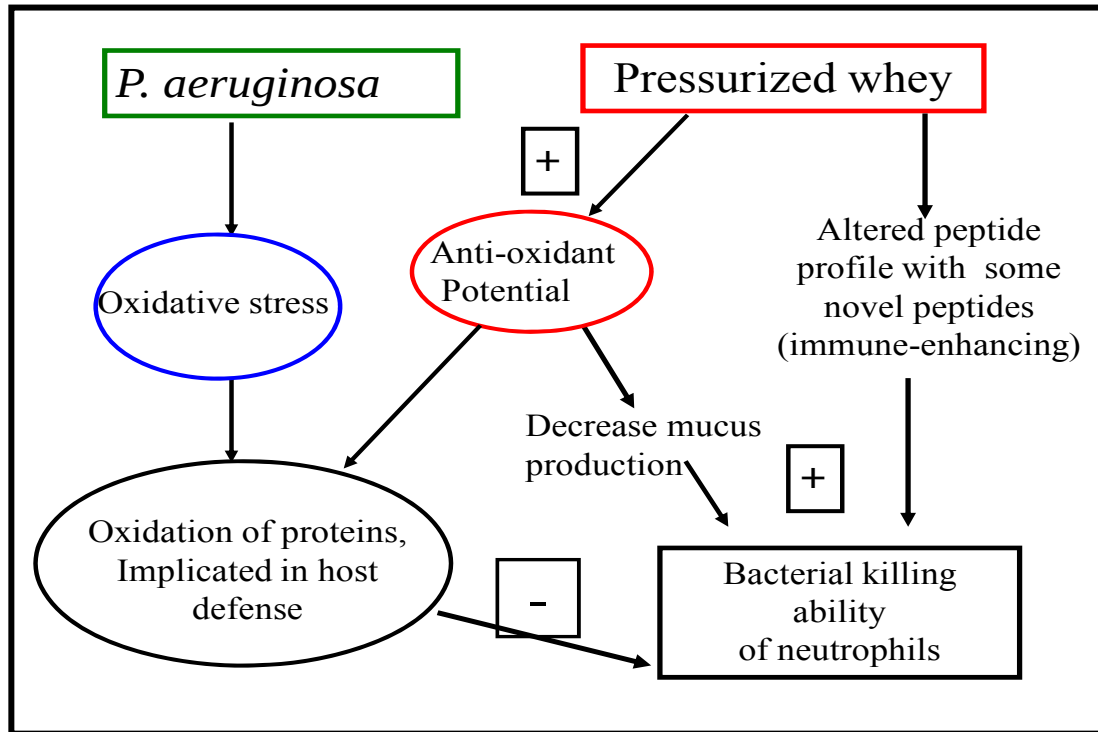


Figure 7.1: Diagram illustrating potential effects of pressurized whey protein on *P. aeruginosa* prolonged lung infection. The altered peptide profile and expected higher efficiency of digestion and hence altered amino acid/peptide profile available for intestinal absorption may result in an immune-enhancing effect in the pressurized whey-fed group. The antioxidant potential of pressurized whey may also protect proteins implicated in host defense, such as surfactant protein A, D, and protect neutrophils from oxidant-induced apoptosis, and/or result in decreased mucus production, which could indirectly enhance bacterial clearance.

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